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(54) Title: ID-1 AND ID-2 GENES AND PRODUCTS AS DIAGNOSTIC AND PROGNOSTIC MARKERS AND THERAPEUTIC TARGETS FOR TREATMENT OF BREAST CANCER AND OTHER TYPES OF CARCINOMA

(57) Abstract: A method for detection and prognosis of breast cancer and other types of cancer. The method comprises detecting expression, if any, for both an Id-1 and an Id-2 genes, or the ratio thereof, of gene products in samples of breast tissue obtained from a patient. When expressed, Id-1 gene is a prognostic indicator that breast cancer cells are invasive and metastatic, whereas Id-2 gene is a prognostic indicator that breast cancer cells are localized and noninvasive in the breast tissue.

Id-1 AND *Id-2* GENES AND PRODUCTS AS DIAGNOSTIC AND PROGNOSTIC MARKERS AND THERAPEUTIC TARGETS FOR TREATMENT OF BREAST CANCER AND OTHER TYPES OF CARCINOMA

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns a diagnosis, prognosis and treatment of breast, endometrium, cervical, ovarian, squamous cell, prostate and melanoma cancer. Particularly, the invention concerns the use of *Id-1* and/or *Id-2* genes or Id-1 and/or Id-2 products as diagnostic markers for cancer cells metastatic aggressivity and use of detection of the *Id-1* or *Id-2* genes, or a ratio thereof, or use of detection of the Id-1 or Id-2 products, or a ratio thereof, for diagnosis and prognosis of breast cancer. The invention further concerns a method for treatment of breast cancer by targeting *Id-1* or *Id-2* genes, or a combination thereof, through delivery of antisense transcripts, ribozymes, small therapeutically active molecules, drugs, peptides or organic compounds that disrupt Id-1 protein interaction with bHLH transcription factor or enhance Id-2 action with bHLH transcription factor and vice versa, RNA, anti-*Id-1* RNAi causing degradation of homologous *Id-1* mRNAs, Id-2 as a gene or a protein, or ITF-2 as a gene or protein, or targeting Id-1 or Id-2 proteins with antibodies or with compounds which either enhance or impair their expression thereby affecting the feedback of the gene expression. The invention further concerns the detection of Id-1 or Id-2 products or genes or their ratio with a kit comprising anti Id-1 and/or Id-2 antibodies or *Id-1* or *Id-2* probes.

Description of Related Art

Breast cancer is one of the most common malignancy among

women and shares, together with lung carcinoma, the highest fatality rate of all cancers affecting females.

There are very few diagnostic markers available for breast cancer detection and those which are available have a predictive accuracy only about twenty percent. There is no marker available that can detect or determine cancer cells metastatic aggressivity.

The current treatment of the breast cancer is limited to a very invasive, total or partial mastectomy, radiation therapy, or chemotherapy, later two resulting in serious undesirable side effects.

It would thus be desirable to have available additional new diagnostic methods which would detect the presence of cancer with greater accuracy and which would permit determination of distinction of highly aggressive breast cancer cells having a tendency to metastasize from the cancer cells which remain localized and have low probability of metastatic spread. It would also be desirable to have available methods for less invasive treatment of the breast or other cancers.

The mammary gland is one of the few organs that undergo striking morphological and functional changes during adult life, particularly during pregnancy, lactation, and involution.

When normal epithelial breast cells become transformed, a number of genetic alteration occur which lead to tumorigenesis and metastasis. These alteration affect growth control, maintenance of differentiated epithelial functions and invasiveness. Identifying the genes involved in these processes is essential for understanding how breast cancer develops, and for deriving better methods for prognosis and treatment.

In both humans and mice, fetal virgin adult, and pregnant mammary glands undergo extensive temporal, structural and

spatial remodeling, which entails invasion, migration, and relocation of cells to generate the ductal and alveolar structures of the gland. Once lactation is terminated, there is additional and extensive tissue remodeling as the gland returns to its resting state.

During each menstrual cycle, and especially during pregnancy, lactation and involution, mammary epithelial cells go through cycles of proliferation, invasion, differentiation and apoptotic cell-death. The mechanisms that regulate these complex and developmentally coordinated cell phenotypes are only poorly understood. However, at least some of the downstream genes that are regulated during these different stages of mammary development have been identified.

In recent years, some progress has been also made in elucidating the mechanisms that regulate mammary gland-specific gene expression and the transformation of mammary epithelial cells to malignancy. However, the practical use of these findings for detection, prognosis and treatment of cancer and its malignant propensities has not been described.

It is, therefore, a primary objective of this invention to provide a method and means for detection and prognosis of breast cancer, for determination of the malignant aggressivity of cancer cells and for providing therapeutically effective agents for suppression and therapy of breast, endometrium, cervical, ovarian, squamous cells and prostate cancer and melanoma.

All patents, patent applications and publications cited herein are hereby incorporated by reference.

SUMMARY OF THE INVENTION

One aspect of the current invention is a method for diagnosis, prognosis and treatment of breast, cervical, ovarian, endometrium, squamous, prostate and melanoma cancer.

Another aspect fo the current invention is the use of *Id-1* and/or *Id-2* genes as diagnostic markers for metastatic

aggressivity of breast, cervical, ovarian, endometrium and squamous cancer cells.

Yet another aspect fo the current invention is the use of Id-1 and/or Id-2 proteins as diagnostic markers for metastatic aggressivity of prostate and melanoma cancer cells.

Still another aspect of the current invention is a method for detection of the *Id-1* or *Id-2* genes, or a ratio thereof, or for detection of the Id-1 or Id-2 products, or a ratio thereof, as the markers for diagnosis and prognosis of breast cancer.

Still yet another aspect of the current invention is a method for treatment of breast cancer and other types of cancer by targeting *Id-1* and/or *Id-2* genes, or a combination thereof, through a delivery of antisense transcripts, ribozymes, small therapeutically active molecules, drugs, peptides or organic compounds that disrupt Id-1 interaction with a bHLH transcription factor or enhance *Id-2* protein action with a bHLH transcription factor, *RNA*, anti-*Id-1* RNAi causing degradation of homologous *Id-1* mRNAs, *Id-2* as a gene or a protein, or *ITF-2* gene or protein.

Yet another aspect of the current invention is a kit for detection of *Id-1* or *Id-2* genes or Id-1 or Id-2 products, or their ratio, said kit comprising anti Id-1 and/or Id-2 antibodies or anti *Id-1* and/or *Id-1* probes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Northern blot showing a pattern of Id-1 and Id-2 expression in the mouse mammary gland.

Figure 2 is a Northern mRNA blot of Id-1 expression in cultured breast cancer cells that were either growing in 10% serum (G) or incubated in serum-free medium (SF).

Figure 3 is a Western analysis using a polyclonal antibody against human Id-1 protein with cross-reactive bands around M_R 40,000 (40 Kda) indicating loading and transfer

efficiency with nine cell clones of T47D-*Id-1*.

Figure 4 is a graphical representation of Boyden Chamber invasion assay for T47D-Id-1 cell clones.

Figure 5 is an autoradiogram of T47D cells incubated with [^{3}H]thymidine.

Figure 6A is a Western analysis of an Id-1 protein expression probed with the Id-1 antibody in non-invasive cancer T47D (lane 1) and metastatic cancer MDA-MB-231 (lane 2) cells. The position of Id-1 protein is indicated. Figure 6B is an immunohistogram wherein panels (a), (b), and (c) are representative sections from ductal carcinomas *in situ* (DCIS), and panels (d), (e), and (f) are Grade 3 invasive carcinomas analyzed by immunohistochemistry with anti Id-1 anti-serum.

Figure 7 is a Northern blot showing *Id-2* mRNA expression in human breast cancer cell lines cultured in serum-free medium for two days.

Figure 8 is a Western analysis showing inverse correlation between Id-1 and Id-2 protein expression in growing (G) and differentiated (Diff) mouse mammary SCp2 epithelial cells in culture.

Figure 9 is a Northern mRNA analysis showing an inverse correlation between Id-1 and Id-2 mRNA expression in growing (G), serum starved (SSt) and laminin-treated mouse mammary SCp2 cells in culture for 24 and 48 hours.

Figure 10A shows reduction of β-casein expression in mammary epithelial cells treated with Id-2 antisense oligonucleotides. Figure 10B shows increase of β-casein expression in mammary epithelial cells infected with a LXSN-*Id-2*-sense and *Id-2*-antisense expression vectors.

Figure 11 is a Northern mRNA analysis displaying a different pattern of expression in the mouse mammary gland *in vivo* at different stages of development wherein V indicates virgin, P indicates pregnant and L indicates lactation stage.

Northern analysis was performed using cDNA probes for mouse β-casein, *Id-1* and *Id-2*.

Figure 12A is a Northern analysis of *Id-1* and *Id-2* mRNA expression in human breast cancer cell lines. Cell were cultured in serum-free medium for 48 hours before RNA was extracted. Lane 1: T47D; lane 2: MCF-7; lane 3: MDA-MB-231 and lane 4: MDA-MB-436 cell lines. Figure 12B shows Id-1 and Id-2 expression in MCF-7 growing in 10% FBS (lane 1) and MCF-7 cultured in serum-free medium for 24 hours (lane 2).

Figure 13 is a Western blot showing Id-1 protein expression. Lane 1 shows MDA-MB436 controls, lane 2 shows MDA-MB436 Id-1 sense infected with an amphotropic retrovirus and lane 3 shows MDA-MB436 Id-1 antisense infected with an amphotropic retrovirus.

Figure 14 is graphical illustration of Figure 13 showing a conversion of aggressive MDA436 cells into non-aggressive cells when treated with *Id-1* antisense amphotropic retrovirus in an *in vitro* invasion assay.

Figure 15 is a graph showing decrease in tumor number in 4T1/BalbC mice treated with various constructs *in vivo*.

DEFINITIONS

As used herein:

"Id" means inhibition of differentiation or DNA binding.

"Id proteins" means proteins which are inhibitors of differentiation or DNA binding. Since Id proteins function by binding basic helix-loop-helix (bHLH) transcription factors, Id-1- or Id-2-interacting proteins are important transcriptional regulators of mammary epithelial cell properties.

"Id-1" means protein expressed by *Id-1* gene. High levels of Id-1 protein are expressed by aggressive and metastatic breast, cervical, ovarian, endometrium and squamous cancer cells. High levels of Id-1 protein are expressed in

noninvasive prostate cancer and melanoma.

"Id-2" means protein expressed by *Id-2* gene. Increased levels of Id-2 protein are crucial for normal breast development. Breast, cervical, endometrium and squamous cancer cells producing high level of Id-2 protein are less invasive. Increased levels of Id-2 protein are expressed by highly invasive and metastatic prostate cancer cells.

"Id-1-interacting proteins" are proteins which interact with Id-1 protein. These proteins are, therefore, important transcriptional regulators of mammary epithelial cell properties.

"ITF-2" is a bHLH transcription factor which interacts with Id-1 and is, therefore, an example of Id-1 interacting protein. ITF-2 appears to be constitutively expressed in SCp2 epithelial cells. Although Id-1 expression fluctuates during mammary epithelial cell growth and differentiation, the expression of ITF-2, determined by ITF-2 mRNA, in such proliferating and differentiating SCp2 cells, does not fluctuate. The mouse ITF-2 (mITF-2) insert was found to contain a 950 bp open reading frame encoding the bHLH and C-terminal domains of ITF-2, but missing the N-terminal region.

"HLH" means helix-loop-helix.

"bHLH" means basic helix-loop-helix.

"GAPDH" means glyceraldehyde-3-phosphate dehydrogenase.

"DAPI" means 4',6-diamidino-2-phenylindole.

"DCIS" means ductal carcinoma *in situ*.

"EGR" means early growth.

"Gene product" means a protein or mRNA.

"RNAi" means RNA interference process for a sequence-specific post-transcriptional gene silencing of a gene by providing a double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNA are the mediators of sequence-specific mRNA degradation.

DETAILED DESCRIPTION OF THE INVENTION

The current invention is based on findings that *Id-1* and *Id-2* genes are involved in tumor progression of breast, cervical, ovarian, endometrium, squamous cells and prostate carcinoma and melanoma and that *Id-1* and *Id-2* genes are involved in the development of breast cancer and are, therefore, suitable to serve as diagnostic markers and therapeutic targets for these types of cancer.

Specifically, it has been discovered that *Id-1* gene is involved in and plays a critical role in the development of a proliferative and invasive phenotype in breast, cervical, endometrium and squamous epithelial cells and that it is constitutively expressed in the least differentiated and highly aggressive human cancer cells and that *Id-2* gene is involved in development of a less aggressive or non-aggressive phenotype in these cancer cells.

The *Id-2* gene, on the other hand is involved in the development of a proliferative and invasive phenotype in prostate cancer cells, where *Id-1* gene seems to play just the opposite role, that is, it is involved in the development of a less aggressive or nonaggressive phenotype and prostate and melanoma cancer cells.

It has been further discovered that both *Id-1* and *Id-2* genes, Id-1 and Id-2 proteins, and their respective ratios, may be conveniently detected.

Additionally, the invention is based on findings that both *Id-1* and *Id-2* genes expression may be effectively suppressed or at least decreased by the targeted conversion with amphotropic retrovirus carrying *Id-1* or *Id-2* antisense.

Consequently, the invention concerns, in its broadest scope, a diagnosis, prognosis and treatment of breast, endometrial, cervical, ovarian, squamous cells or prostate carcinoma or melanoma.

I. Function of *Id-1* and *Id-2* Genes and Breast Cancer

Aggressive breast cancer cells that are metastasizing to other parts of the body have been known to loose a specific regulation of the gene involved in normal breast cell development. By contrast, normally developing breast cells maintain this regulation. Little is known, however, about the transcriptional regulators that control the expression of these developmental stage-specific genes.

Basic helix-loop-helix (bHLH) transcription factors are key regulators of lineage- and tissue-specific gene expression in a number of mammalian and non-mammalian organisms. These transcription factors bind DNA as homo- or heterodimers, and activate the transcription of target genes containing E-boxes or E-box-like sequences in their promoters. Dimerization occurs through the HLH domains, whereas DNA binding occurs through the two basic domains.

Id proteins, which are inhibitors of differentiation or DNA binding, are helix-loop-helix (HLH) proteins that lack a basic domain. Id proteins act as dominant inhibitors of bHLH transcription factors by forming transcriptionally inactive heterodimers.

So far, four Id genes (Id-1 through Id-4) have been identified. These genes, although similar in their organization and HLH sequences, localize to different chromosomes and show differences in their pattern of expression and function. For example, the cytogenetic location of Id-1 protein is 20q11, whereas location of Id-2 is 2p25, location of Id-3 is 1p36.13-p36.12 and location of Id-4 is 6p22-p21.

The helix-loop-helix protein Id-1 has been shown to inhibit the activity of basic helix-loop-helix transcription factors, and is an important regulator of cell growth and tissue-specific differentiation.

These findings led inventors to investigate a possible

correlation between the levels of Id-1 protein and the aggressiveness of human breast cancer cells leading to the current discovery.

A. *Id-1* and *Id-2* Gene DNA Sequences

Nucleotide sequences of human *Id-1* and *Id-2* genes are known and have been deposited at GenBank under Accession numbers D13891 and X77956, respectively. Nucleotide sequence which is a source for *Id-1* gene comprises of 926 nucleotides with an *Id-1* gene encoding region starting at nucleotide 36 and ending at nucleotide 500. Nucleotide sequence which is a source for *Id-2* gene comprises of 1049 nucleotides with *Id-2* gene coding region starting at nucleotide 97 and ending at nucleotide 501.

B. Function of Id-1 and Id-2 Genes

It has been now discovered that *Id-1* and *Id-2* genes function as negative regulators of helix-loop-helix (bHLH) transcription factors playing a critical role in the development of a proliferative and invasive phenotype. Such function of *Id-1* and *Id-2* genes was not previously known.

During the development of the current invention the ectopic expression of the *Id-1* gene has been found to inhibit differentiation and stimulate the proliferation and invasiveness of mammary epithelial cells.

The expression of *Id-2* gene, on the other hand, has been found to be up-regulated during differentiation of mammary epithelial cells and its expression increased in the differentiated human breast cancer cells. Such up-regulation of *Id-2* expression was found to be a necessary step toward a fully differentiated phenotype in breast cells.

Compared to expression of *Id-1*, expression of *Id-2* was found to be much higher in the differentiated human breast cells than the expression of the very aggressive and metastatic cells leading to conclusion that there may be a

correlation between the levels of Id-1 or Id-2 proteins and the aggressiveness or non-aggressiveness in human breast cancer cells.

The Id-1 and Id-2 protein levels change dramatically at different stages of breast development. An increase in the level of Id-2 protein is crucial for normal breast development. In breast cancer cells, the cancer cells producing high levels of Id-2 protein are less invasive. By contrast, aggressive and metastatic breast cancer cells express high level of Id-1 mRNA and Id-1 protein.

c. Experimental Evidence and Studies

The evidence supporting the above described findings is based on studies performed on murine epithelial cell lines, on normal mouse mammary glands *in vivo*, on human breast cancer cells and on human breast cancer biopsies.

II. Effect of Manipulating Id-1 Expression on Differentiation of Murine Mammary Epithelial SCp2 Cell Phenotypes

SCp2 cells, a cell line developed from murine mammary gland, are a useful model system for studying mammary epithelial cell growth and differentiation in cell culture.

A role of Id genes in the normal differentiation of SCp2 cells was first suggested by inventors prior findings that Id-1 expression declined rapidly to undetectable levels when the cells differentiated in response to lactogenic hormones, such as insulin, prolactin and hydrocortisone and upon contact with basement membrane (Mol. Cell Biol., 15:3398-3404 (1995).

To directly test the role of Id-1 in these cells, the cells were transfected with an expressible murine Id-1 gene, in either the sense or antisense orientation.

In monolayer culture and low serum medium, Id-1 sense cells grew faster than control cells transfected with the vector lacking a cDNA insert. By contrast, Id-1 antisense cells grew more slowly than controls. Both Id-1 sense and Id-

1 antisense cells ceased growth and formed aggregates or spheres when provided with basement membrane and lactogenic hormones. However, Id-1 sense cells formed spheres that were less compact than spheres formed by controls or antisense expressing cells, and failed to express the milk protein ß-casein. Under the same conditions, Id-1 antisense cells expressed ß-casein at a higher level than control cells.

Despite differences in ß-casein expression, control, Id-1 sense and Id-1 antisense cells exposed to hormones and basement membrane remained a growth arrested for 5 to 6 days. After 8-10 days, however, spheres of Id-1 sense cells began to disintegrate as individual cells dissociated from the sphere, began to invade the basement membrane and resumed growth. In the Boyden Chamber invasion assay, Id-1 sense cells were much more invasive than normal SCp2 or Id-1 antisense cells. The Id-1 sense cells, unlike control or Id-1 antisense cells, expressed a gelatinase of approximately 120 kDa. The activity of this gelatinase was specifically inhibited by inhibitors of matrix-metalloproteinases.

Id-1 protein expression in the nontransformed SCp2 cells resulted in a loss of cell-cell interaction, loss of ability to express markers of differentiation and in an increased ability to invade a basement membrane, migrate and proliferate.

All these propensities make the cells expressing constitutively high levels of Id-1 protein most highly aggressive and metastatic.

2. <u>The Role of Id-1 in Normal Mammary Gland Development *In Vivo*</u>

The role of Id-1 in normal mammary gland was determined by following the expression of Id-1 during normal mouse mammary gland development *in vivo*, using Northern analysis of total RNA from virgin (V), pregnant (P; days 2 to 18), and lactating (L) mice. Result are shown in Figure 1.

Figure 1 is a Northern analysis of total RNA extracted from mouse mammary gland at different stages of development. Northern analysis utilized cDNA probes for mouse β-casein, *Id-1* and *Id-2* gene expression.

As seen in Figure 1, β-casein mRNA was evident only during mid and late pregnancy and lactation. When the blot was reprobed with *Id-1* cDNA, Id-1 expression was found to be inversely correlated with β-casein expression, confirming the role of *Id-1* gene *in vivo* observed in the SCp2 cells.

These results clearly show that Id-1 expression declines when the mammary gland proceeds toward full differentiation during pregnancy and at the lactation stage. Id-1 thus is expressed primarily in cells which are nondifferentiated or begin to differentiate.

3. Analysis of Id-1 Expression in Human Breast Cancer Cell lines and Breast Biopsies

Findings that ectopic Id-1 expression induced an invasive phenotype in mouse mammary epithelial cells suggested that *Id-1* gene could contribute to human breast cancer progression.

To begin to explore this possibility, human breast cancer cell lines exhibiting varying degrees of invasiveness in culture and *in vivo*, using metastatic tumor formation in nude mice, was examined. Results of these studies show that highly aggressive human breast cells have lost their serum regulation of *Id-1* gene expression. Results are shown in Figures 2-6.

The regulation of *Id-1* gene expression in the presence of serum was examined in non-invasive cancer T47D and MCF-7 cell lines and in aggressive and invasive cancer MDA-MB-231 and MDA-MB-435 cell lines. The first two are noninvasive human breast cancer cell lines, the latter two are highly invasive metastatic cells which were selected for a highly aggressive phenotype by passage in immunodeficient mice. All cells were purchased from the American Tissue Culture Collection (ATCC).

In some cells, *Id-1* gene expression is known to be induced by certain mitogens, such as, for example, serum. Consequently, the effect of the presence or absence of serum on expression of *Id-1* gene in these two types of cells was investigated. RNA was isolated from both types of cells that were grown on either 10% serum (G) or incubated in serum-free medium (SF). RNA was then analyzed by Northern analysis according to Example 3. Results are seen in Figure 2.

Figure 2 illustrates a loss of serum-regulated *Id-1* expression in aggressive breast cancer cells. Upper panel shows a position of *Id-1* mRNA (1.2 kb). Lower panel shows a position of the ribosomal 28S RNA used as control for RNA integrity and quantitating.

As seen in Figure 2, T47D and MCF-7 non-invasive cancer cells expressed high levels of *Id-1* mRNA only when cultured in serum. When cultured in serum-free medium for two days, such expression levels were undetectable. In contrast, highly aggressive and metastatic MDA-MB-231 and MDA-MB-435 cells constitutively expressed *Id-1* mRNA, regardless of the presence or absence of serum.

These results show that in non-invasive breast cancer cell, the expression of *Id-1* gene could be induced by culturing these cells in the presence of serum. However, in these non-invasive breast cancer cells, this gene was not expressed and the expression could not be induced in serum-free medium. On the contrary, the invasive metastatic cancer cells expressed *Id-1* gene in both the serum containing and serum-free medium. Consequently, the invasive metastatic breast cancer cells do not need *Id-1* expression induction by serum but it is in their cellular make-up to express *Id-1* gene constitutively.

4. Constitutive Id-1 Expression Converts a Nonaggressive into a More Aggressive Breast Cancer Cell Line

To test whether the unregulated *Id-1* expression contributes to aggressive phenotype of human breast cancer cells and to determine if the induced constitutive *Id-1* expression would convert a nonaggressive cells into an aggressive metastatic cells, constitutive *Id-1* expression was investigated.

For this purpose, the human *Id-1* cDNA was expressed in nonaggressive T47D cells using amphotropic retrovirus (pBabe-*Id-1*). Production of pBabe-*Id-1* retroviral vector and virus are described in Example 1. Retroviral infection is described in Example 2. Puromycin was used to select virus-expressing cells.

Briefly, approximately eight RT-units of either pBabe-puro or pBabe-Id-1 retrovirus were mixed with 5 ml of medium containing 4 μg/ml polybrene and were added to T47D cells in 100-mm dishes. Cells expressing the retroviral genes were selected in 0.6 μg/ml puromycin, which killed all of the mock-infected cells within three days, whereas 80 or 30% of the pBabe-puro or pBabe-Id-1-infected cells, respectively, survived. The puromycin-resistant cells are referred to as T47D-pBO or T47D-Id-1. To establish single-cell clones, the T47D-Id-1 population was plated at 1-2 cells/well in 24-well tissue LXSN retroviral vector was prepared in the same way except neomycin was used to select virus expressing cells culture plates. Clones that grew in the wells were expanded. Results are seen in Figure 3.

Figure 3 illustrates Id-1 protein levels obtained in nine clones. When T47D cells were infected with pBabe-*Id-1* retrovirus, nine single-cell-derived clones (clone 1-clone 9) were obtained. The clones were cultured in serum-free medium for two days before protein extraction and Western analysis

using a polyclonal antibody against human Id-1. Positions of Id-1 protein and molecular weight markers in each clone are indicated. Cross-reactive bands around M_r 40,000 (40kDa) indicate loading and transfer efficiency.

From nine single-cell-derived clones isolated from the T47D-Id-1 population, the clone 6 was lost during processing. Each of the eight surviving clones expressed a different level of Id-1 protein, as determined by Western analysis. Clones 1, 2, and 8 expressed relatively high levels of Id-1 protein in serum-free medium, whereas clones 4 and 9 expressed very low levels of Id-1 under these conditions. The other clones expressed Id-1 at intermediate levels.

Five T47D-Id-1 clones, expressing either high or low levels of Id-1 in serum-free medium, were then examined for invasiveness using the Boyden Chamber invasion assay. Conditions of the Boyden Chamber invasion assay are described in Example 5. Results are shown in Figure 4.

Figure 4 illustrates Boyden Chamber invasion assay for T47D clones. Cells were cultured in serum-free medium for 2 days before they were placed in the upper chamber of Matrigel-coated *trans*-well filters. The invasion assay was carried out for 20 hours in serum-free medium and cells that migrated through the filter were stained and counted. Results were averaged and SDs were calculated.

As seen in Figure 4, the invasive activity of each clone was approximately proportional to the level of Id-1 protein expression. Thus, clones with constitutively high levels of Id-1 (clones 1, 2, and 8) were more invasive then clones expressing low levels of Id-1 protein (clones 4 and 9). The invasive activity of the low-expressing clones resembled that of the uninfected parental T47D cells (not shown).

Ectopic Id-1 expression also conferred a growth advantage in serum-free medium, as measured by the percentage of cells incorporating [^{3}H]-thymidine. Conditions of the [^{3}H]-thymidine

labeling are described in Example 6. Results are seen in Figure 5.

Figure 5 shows percentage of labeled nuclei of cells cultured in serum-free medium for 32 hours before incubation with [^{3}H]-thymidine for additional 16 hours and processed by autoradiography. Cell that incorporated [^{3}H]-thymidine were calculated as a percentage of total DAPI-stained nuclei.

As seen in Figure 5, the three T47D-*Id-1* clones that expressed Id-1 proteins at higher levels had a greater [^{3}H]-thymidine-labeling index than two clones in which Id-1 expression was lower. The three T47D-*Id-1* clones that expressed Id-1 protein at higher levels had a greater thymidine-labeling index than two clones in which expression of Id-1 protein was lower. Thymidine-labeling index for clones 1, 2 and 8 was 59%/average, for clones 4 and 9 it was 36%/average.

These results show that when normal Id-1 regulation is lost and Id-1 is constitutively expressed, human breast cancer cell lines acquire increased invasiveness and a proliferative advantage in a growth factor-deficient media. Ectopic Id-1 expression converted a relatively nonaggressive breast cancer cell line into a relatively aggressive one.

These results show that by determining a level of Id-1 protein expression, evaluation of the breast cells aggressivity can be made.

Since the above findings indicated that Id-1 expression may serve as a prognostic marker for certain subset of aggressive breast cancers, breast cancer biopsies for Id-1 expression were further examined by immunohistochemistry.

5. <u>Id-1 Expression in Breast Cancer Biopsies</u>

To determine whether the above obtained observations are applicable to humans, a large number of breast cancer biopsies were obtained from patients and immunohistochemical reactions as well as Western analyses were performed.

Immunohistochemical determination of the expression of Id-1 protein was carried out on a total of eighty-three breast cancer biopsies obtained from patients treated at California Pacific Medical Center.

Twenty-three of the biopsies were ductal carcinoma *in situ* (DCIS), sixty biopsies were infiltrating carcinomas of which twelve were Grade 1, seven were Grade 2 and forty-one were of Grade 3 carcinoma.

Out of twenty-three ductal carcinomas in situ (DCIS), 18 were found negative (78%), three were weakly positive (13%), and two were strongly positive (9%). Infiltrating carcinomas Grade 1, which is the least aggressive amongst the invasive tumors, displayed a pattern of Id-1 protein expression similar to the DCIS. Out of twelve Grade 1 carcinoma, 10 were negative (83%), 1 was weakly positive (8.5%), and 1 was strongly positive (8.5%). On the other hand, the majority of the infiltrating Grade 2 and Grade 3 carcinomas, the most aggressive tumors, were weakly or strongly Id-1 positive. Out of seven Grade 2 carcinomas, 3 were negative, 1 was weakly positive, and 3 were strongly positive. Out of forty-one Grade 3 carcinomas, 16 were negative (39%), 4 were weakly positive (10%), and 21 were strongly positive (51%).

Results are seen in Table 1.

TABLE 1

Id-1 Protein Expression Determined By Immunohistochemistry in 83 Breast Cancer Biopsies

Tumor Type	Id-1 Negative	Id-1 Weakly Positive	Id-1 Strongly Positive
Ductal Carcinoma *in Situ*	78% (18/23)	13% (3/23)	9% (2/23)

Infiltrating Carcinoma			
Grade 1	83% (10/12)	8.5% (1/12)	8.5% (1/12)
Grade 2	43% (3/7)	14% (1/7)	43% (3/7)
Grade 3	39% (16/41)	10% (4/41)	51% (21/41)

Numbers in parenthesis indicate the actual number of biopsies out of the total number of biopsies examined.

Results of six selected representative samples in this assay are seen in Figures 6A and 6B which show expression of Id-1 in human breast cancer biopsies. Immunohistochemistry was carried out using a specific batch of anti-Id-1 antibody, confirmed by Western analysis to show no cross-reactive bands. Immunohistochemical procedure is described in Example 8.

Figure 6A is a Western analysis showing the specificity of the Id-1 antibody used for immunohistochemistry. Lane 1 shows non-invasive T47D cancer cells, lane 2 shows invasive and metastatic MDA-MB-231 cancer cells. All cells were cultured in serum-free medium for 48 hours. Position of Id-1 protein is indicated. No cross-reactive band is seen. Results shown in Figure 6A clearly confirm high expression of Id-1 protein in the cancer cells when compared to Id-1 expression in T47D cells.

Figure 6B shows representative section from DCIS (panels a, b, and c) and Grade 3 invasive carcinoma (panels d, e and f) which were analyzed by immunohistochemistry using antiserum against Id-1 protein. The majority of DCIS were negative (panels a and b), one showed strong positivity in its large ductal structure (panel c). The majority of infiltrating carcinoma, on the other hand, showed strong Id-1 immunoreactivity (panel d and e). Minority of the invasive tumors were negative (panel f). In panel d, a differentiated glandular section, the structure with the lumen was negative whereas infiltrating cells showed strong immunoreactivity.

These results show that almost all examined ductal carcinomas in situ (DCIS) were negative for Id-1 staining. However, the majority (51%) of infiltrating Grade 3 carcinomas of ductal origin were strongly Id-1 positive. These results confirm that Id-1 is a reliable prognostic marker for breast cancer invasiveness and metastatic propensity.

6. Expression of Id-2 in Human Breast Cancer Cells

To determine if the expression of Id-1 protein was specific to aggressive malignant cancer cells or if this was common property of Id proteins, the expression of the second Id protein, namely Id-2 protein, in human breast cancer cells was examined.

Id-2 expression in human cancer cells was determined by Northern analysis. The same four types of cells were used as used previously in studies with Id-1. These cells were cultured in serum-free medium for two days before RNA was extracted. The blot was hybridized with a human Id-2 cDNA probe. Results are shown in Figure 7.

Figure 7 is a Northern analysis of Id-2 transcripts. Upper panel shows expression of Id-2 mRNA in non-invasive T47D (lane 1) and MCF7 (lane 2) cancer cells and in highly aggressive, metastatic and invasive MDA-MB-231 (lane 3) and MDA-MB436 (lane 4) cancer cells. Lower two panels show a positions of two ribosomal 28S and 18S RNA used as control for RNA integrity.

Figure 7 shows that under the same experimental conditions as those described for Id-1, Id-2 mRNA was found to be expressed in lanes 1 and 2, which correlate with non-invasive T47D and the MCF7 human breast cancer cell lines. As seen in Figure 7, lanes 3 and 4, there was no detectable Id-2 mRNA in lanes 3 or 4, which represent highly invasive MDA-MB-231 and MDA-MB-436 human breast cancer cell lines.

Thus, in contrast to Id-1, the expression of Id-2 gene products, such as the protein and mRNA, correlates with non-

aggressive or non-invasive cancers.

These results show that both Id-1 and Id-2 are fair indicators of breast cancer presence and aggressivity and that each indicates and is found in a different type of cancer cells. Detection of Id-1 expression indicates presence of highly aggressive, metastatic and invasive cancer cells. Detection of Id-2 expression indicates presence of noninvasive cancer cells.

7. Inverse Correlation between Id-1 and Id-2 Expression

A direct regulatory link has been found to exist between *Id-1* and *Id-2* genes in breast cells. Id-2 protein expression is generally high when Id-1 protein expression is low, both *in vitro* and in vivo, confirming an existence of a negative correlation in expression levels.

a. Id-2 Expression *In Vitro*

To determine the pattern of Id-2 expression during mammary cell growth and differentiation, expression of Id-2 protein during mammary epithelial cell differentiation *in vitro* and *in vivo* was undertaken.

For this purpose, the yeast two-hybrid system and the basic helix-loop-helix protein ITF-2 as a bait were used to isolate Id-2 from a library derived from differentiated, milk-producing mammary epithelial cells. First, Id-2 protein expression in SCp2 cells during proliferation or differentiation was investigated, using Western analysis. Results are shown in Figure 8.

Figure 8 is a Western analysis showing inverse correlation between Id-1 and Id-2 protein expression in growing (G) and differentiated (Diff) SCp2 mammary epithelial cells treated with Matrigel and lactogenic hormones for 48 and 72 hours. Protein was extracted and analyzed using antibodies specific for Id-1, Id-2 and β-casein milk protein, which is the marker for mammary epithelial cells differentiation.

As shown in Figure 8, differentiated cells expressed high levels of the Id-2 (16 kDa) protein, similarly to expression of β-casein, at both 48 and 72 hours. In comparison, Id-1 protein was detectable only in proliferating cells (lane G). No expression of Id-1 protein was detected in differentiated cells. These results clearly show that there is an inverse correlation between Id-1 and Id-2 protein.

To confirm this inverse correlation between Id-1 and Id-2 expression, Northern analysis of SCp2 cells proliferating or treated with laminin for 24 and 48 hrs was performed. Laminin is an important component of extracellular matrix and can trigger differentiation. Results are seen in Figure 9.

Figure 9 is a Northern analysis of inverse correlation between Id-1 and Id-2 mRNA expression in growing (G), serum starved (SSt), and Laminin-treated SCp2 mammary epithelial cells for 24 and 48 hours. Total RNA was extracted and analyzed using probes specific for Id-1, Id-2 and β-casein.

Results seen in Figure 9 confirm results seen in Figure 8. There was expression of both Id-2 and β-casein in differentiated cells, but there was no expression of Id-1 in these cells. Id-1 was expressed only in growing (G) cells confirming that the inverse correlation exists between expression of Id-1 and Id-2 mRNA.

In order to determine if Id-2 up-regulation was a crucial event for mammary epithelial cell differentiation and milk production, two sets of experiments were performed. In the first set, SCp2 cells were treated with Laminin and lactogenic hormones for 48 hrs in the presence of either control oligonucleotides or Id-2 antisense oligonucleotides. Results are seen in Figure 10.

Figure 10A illustrates reduction of β-casein expression in mammary epithelial cells treated with Id-2 antisense oligonucleotides. Lane 1 shows SCp2 cells treated with Laminin for 48 hours and control oligonucleotide. Lane 2 shows Scp2

cells treated with Laminin for 48 hours and with Id-2 oligonucleotide. Figure 10B illustrates increase of β-casein expression in mammary epithelial cells infected with a LXSN-Id-2 sense expression vector (Lane 2) and inhibition of β-casein expression in cells infected with a LXSN-Id-2 antisense expression vector (Lane3). Lane 1 corresponds to cells infected with a LXSN-control vector.

As seen in Figure 10A, a dramatic reduction of ß-casein expression was observed in Id-2 antisense oligonucleotide treated cells. In the second set of experiments, SCp2 cells were infected with either LXSN-control, LXSN-Id2-sense or LXSN-Id2-antisense constructs, selected with neomycin and treated with laminin for 48 hrs. As shown in Figure 10B, ß-casein expression was increased in SCp2-LXSN-Id2-sense cells in comparison to control. Most dramatically, ß-casein expression was almost undetectable in SCp2-LXSN-Id2-antisense cells.

The results seen in Figures 10A and 10B show that Id-2 is involved and necessary in and its up-regulation occurs during mammary cell differentiation. However, the results in Figure 10B also shows that such up-regulating can be effectively negated with Id-2 antisense carrying construct.

b. Id-2 Expression *In Vivo*

To determine Id-2 protein expression *in vivo* and to compare it to the expression of Id-1 protein, another set of experiments was performed.

In these studies, the level of Id-1 and Id-2 mRNA during mammary gland development *in vivo*, using Northern analyses of total RNA from virgins, pregnant and lactating mice were determined. Results are seen in Figure 11.

Figure 11 shows a different pattern of Id-1 and Id-2 protein expression in the mouse mammary gland *in vivo*. Total RNA was extracted from mouse mammary glands at different

stages of development. Northern analyses using sDNA probes for mouse β-casein, Id-1 and Id-2 were performed. V indicates virgin; P indicates pregnant at days 2, 5, 12 and 18 and L indicates lactation mammary gland.

As seen in Figure 11, ß-casein mRNA was evident only during mid and late pregnancy and during lactation. When the blot was then reprobed with a mouse *Id-1* cDNA, *Id-1* mRNA expression resulted. Such *Id-1* expression was inversely correlated with ß-casein expression, suggesting a similar role for Id-1 gene *in vivo* to that observed in the SCp2 cells, that is, Id-1 expression declines when the mammary gland proceeds towards full differentiation as, for example, in lactation stage. On the other hand, expression of *Id-2* mRNA was barely detectable in virgin gland and at the beginning of pregnancy. Its expression increased at day 12 of pregnancy, when epithelial cells start producing the milk protein ß-casein. Id-2 expression was at its highest level toward the end of pregnancy (day 18) and lactation, when the epithelial cells were fully differentiated.

The above results show that the expression pattern of *Id-2* mRNA or gene expression is different from that of *Id-1* mRNA. Id-2 expression level is opposite to that of Id-1 expression during periods of cell growth and differentiation. This further indicates a differentiating role for Id-2, in contrast to Id-1, during mammary gland development.

The terminal development of the mammary gland involves the contribution of proliferative as well as differentiative events. These events must be tightly coordinated. Id-2 as well as Id-1 were shown to play a central role in this regulation by negatively regulating different sets of bHLH proteins. Moreover, the expression of these two genes was found to be tightly coordinated.

c. Analysis of Id-2 Expression in Breast Cancer Cells

To confirm that similar findings to those found in murine mammary epithelial cells *in vitro* and *in vivo*, Id-2 expression was investigated in human breast cancer cell lines in culture using the same mouse Id-2 cDNA probe.

For this purpose, the two T47D and MCF7 cancer cell lines which display non-aggressive and differentiated characteristics in culture (in absence of estrogen), and the two highly aggressive and metastatic MDA-MB-231 and MDA-MB-436 cell lines were used. The cells lines were described above. Results are seen in Figure 12.

Figure 12A is a Northern analysis of Id-1 and Id-2 mRNA expression in human breast cancer cell lines. Cells were cultured in serum-free medium for 48 hours before RNA was extracted and subjected to blotting. Lane 1 shows T47D cancer cell line; lane 2 shown MCF-7 cancer cell line; lane 3 shows MDA-MB-231 cancer cell line and lane 4 shows MDA-MB-436 cancer cell line. Figure 12B shows Id-1 and Id-2 expression in MCF-7 growing in 10% FBS (lane 1) and MCF-7 cultured in serum-free medium for 24 hours (lane 2).

As seen if Figure 12A, when cultured in serum-free conditions for 48 hrs, MCF-7 cells, and to a lesser extent T47D cells, expressed high levels of Id-2 mRNA. However, Id-2 expression was undetectable in the two aggressive cell lines MDA-MB-231 and MDA-MB-436 where, as expected, Id-1 was highly expressed. Id-1 expression was not detected in non-aggressive T47D and MCF-7 cancer cells.

These results again confirm, this time in human breast cancer cells, the inverse correlation between the expression of the two HLH proteins that was previously determined to exist in mammary epithelial cells and imply a different role for Id-2 from Id-1 in breast cancer cell phenotypes. This is seen especially clearly in Figure 12B, where, upon serum-withdrawal, the levels of Id-2 mRNA were found to be increased in MCF-7 cells whereas the levels of Id-1 mRNA were decreased.

All the data presented above clearly show the role of the two helix-loop-helix proteins, Id-1 and Id-2, as molecular switches not only between growth/invasion and differentiation in mammary epithelial cells, but also during breast cancer progression.

8. Targeting Id-1 Reduces Breast Cancer Cell Invasion *In Vitro*

To determine whether the *Id-1* is a key gene which regulates the aggressive phenotype of human breast cancer cells, studies were performed to determine whether Id-1 antisense expression converts a very aggressive and metastatic breast cancer cell into a non-aggressive one.

For this purpose, the human Id-1 cDNA was expressed in a sense as well as an antisense orientation in human metastatic MDA-MB436 breast cancer cells using an amphotropic LXSN-Id-1 sense and antisense retrovirus. Neomycin was used to select for virus-expressing cells. Results are shown in Figure 13.

Figure 13 is a Western analysis of Id-1 expression of highly aggressive and invasive MDA-MB436 cancer cells. Actin was used as control. Lane 1 shows MDA-MB436 cells as control against MDA-MB436 treated with Id-1 sense retrovirus (lane 2) or MDA-MB436 treated with Id-1 antisense infected with retrovirus (lane 3).

As seen in Figure 13, cells infected with a control virus (empty plasmid, lane 1) expressed detectable levels of Id-1 protein in serum-free medium. The LXSN-*Id-1* sense infected population (lane 2) expressed even higher levels of Id-1 protein whereas the LXSN-*Id-1* antisense infected cells (lane 3) expressed very low levels of Id-1 under these conditions.

The same three populations of cells were then tested in a Boyden Chamber invasion assay to compare their ability to migrate and invade a reconstituted basement membrane. Results

are seen in Figure 14.

Figure 14 shows results of the invasion assay where the assays were performed in modified Boyden Chambers assay described in Example 5 with 8 μm pore filter inserts for 24-well plates obtained from Collaborative Research. Filters were coated with 10-12 μl of ice-cold Matrigel (7.3 mg/ml protein) obtained from Collaborative Research. Cells (100,000 per well) were added to the upper chamber in 200 μl of the appropriate medium containing 0.1% bovine serum albumin (BSA). In general, cells were assayed in triplicate or quadruplicate, and the results averaged. The lower chamber was filled with 300 μl of NIH-3T3 cell-conditioned medium according to Cancer Res., 47:3239-3245 (1987). After a 20 hours incubation, cells were fixed with 2.5% glutaraldehyde in PBS and stained with 0.5% toluidine blue in 2% Na_2CO_3. Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy.

The invasive activity of each cell population was proportional to the level of Id-1 protein expression as seen in Western blot shown in Figure 13. The population with high levels of Id-1 (LXSN-Id-1 sense cells, lane 2) was much more invasive than the population expressing low levels of Id-1 (LXSN-Id-1 antisense cells, lane 3). The invasive activity of the control population expressing intermediate levels of Id-1 protein was also intermediate (lane 1).

These results further confirm that the aggressivity and invasiveness of the human breast cancer cells can be attributed to the high expression of *Id-1* gene and also show that aggressivity of cells expressing Id-1 protein can be reduced or eliminated by treatment with an *Id-1* antisense constructs. Consequently, the expression of Id-1 in human breast cancer cells is a good prognostic and diagnostic tool for detection of aggressive breast cancer and for

distinguishing such aggressive and invasive cancer from the non-invasive cancer cells attributable to their expressing Id-2 protein.

9. Targeting Id-1 Reduces Breast Cancer Cell Metastasis *In Vivo*

Following the finding that targeting Id-1 with an antisense comprising construct reduces aggressivity of breast cancer cells *in vitro*, further studies were undertaken to determine if the same would be valid for breast cancer cells *in vivo*, and if the metastatic propensity of cancer cells expressing Id-1 could be changed to nonaggressive cells.

In order to determine the role of *Id-1* in the metastatic process *in vivo*, the 4T1 murine metastatic breast cancer cell line which express, like human MDA-MB231 and MDA-MB436 cells, high levels of *Id-1* mRNA and protein and which metastasize to the lungs were used. In order to deliver the *Id-1* antisense constructs, the technique of cationic liposome-DNA complex (CLDC)-based intravenous gene delivery according to J. Biol. Chem., 274:13338-13344(1999) was utilized. This CLDC-based intravenous (iv) delivery (tail vein injections) of Id-1 antisense construct, such as plasmid, significantly reduced the metastatic spread of 4T1 breast cancer cells in 4T1BalbC mice. Results are seen in Figure 15.

Figure 15 is a graph illustrating a tumor reduction in 4T1/BalbC mice treated with various constructs. Specifically, the mice were treated with luciferase (lane 1), with irrelevant gene serving as another control (lane 2) and with Id-1 antisense (lane 3).

Results shown in Figure 15 clearly show that the number of highly aggressive and metastatic tumor decreases significantly when the tumor cells are targeted with Id-1 antisense construct.

Specifically, a single injection of CLDC containing Id-1

antisense, three days after iv injection of 50,000 4T1 cells, dramatically reduced the total number of lung metastases (lane 3), when compared to tumor-bearing mice treated with CLDC containing control genes (luciferase as well as an irrelevant gene, lanes 1 and 2).

These results show first that the aggressive tumor growth and metastasis can be treated with antisense Id-1 construct and, second, that CLDC-based plasmid antisense delivery, which is a novel delivery approach, is a practical way of achieving such delivery.

10. Cumulative Evidence for *Id-1* and *Id-2* Function in Breast Cancer Aggressivity and Diagnosis and Treatment Thereof

Invention described herein showed that aggressive metastatic breast cancer cells express high levels of *Id-1* mRNA because of a loss of serum-dependent relation that is mediated by the 2.2-kb region of the human *Id-1* promoter. This suggests that unregulated *Id-1* gene expression may be an important regulator of the aggressive phenotype of a subset of human breast cancer cells. The results disclosed herein further implicated *Id-1* gene as a critical downstream target of steroid hormones and critical mediator of the aggressive phenotype in a subset of human breast cancer cells.

Specific findings are as follows:

The Id-1 gene is highly expressed during proliferation, and is down-regulated when mammary epithelial cells differentiate.

The Id-2 gene is not expressed in growing mammary epithelial cells, and is up-regulated during differentiation.

Id-1 expression declines when the mammary gland proceeds toward full differentiation during pregnancy and at the lactation stage. Id-1 thus is expressed primarily in cells which are nondifferentiated or begin to differentiate.

In non-invasive breast cancer cell, the expression of *Id-1* gene can be induced by culturing these cells in the presence of serum. However, in these non-invasive breast cancer cells, this gene is not expressed and the expression cannot be induced in serum-free medium. To the contrary, the invasive metastatic cancer cells express *Id-1* gene in both the serum containing and serum-free medium. Consequently, the invasive metastatic breast cancer cells do not need *Id-1* expression induction by serum but it is in their cellular make-up to express *Id-1* gene constitutively.

The constitutive expression of Id-1 inhibits differentiation of mammary epithelial cells, and induces proliferation and invasion.

Certain aggressive breast cancer cells constitutively express high levels of Id-1 protein, apparently due to the loss of serum-dependent regulation.

The expression of Id-1 directly correlates with the level of aggressiveness in breast cancer cell lines and evaluation of the breast cells aggressivity can be made in breast cancer biopsies by determining a level of Id-1 protein expression. Almost all examined ductal carcinomas in situ (DCIS) were negative for Id-1 staining. However, the majority (51%) of infiltrating Grade 3 carcinomas of ductal origin were strongly Id-1 positive. These results confirm that Id-1 is a reliable prognostic marker for breast cancer invasiveness and metastatic propensity.

The expression of Id-2 directly correlates with the level of differentiation and non-aggressiveness breast cancer cells. Id-2 is involved in and its up-regulation occurs during mammary cell differentiation. Such up-regulating can be effectively negated with Id-2 antisense carrying construct.

Id-1 and Id-2 are fair indicators of breast cancer presence and aggressivity and each indicates and is found in

a different type of cancer cells. Detection of Id-1 expression indicates presence of highly aggressive, metastatic and invasive cancer cells. Detection of Id-2 expression indicates presence of noninvasive cancer cells. The expression pattern of Id-2 protein is different from that of Id-1 protein. Id-2 expression level is opposite to that of Id-1 expression during periods of cell growth and differentiation.

The expression of Id-1 in human breast cancer cells is a good prognostic and diagnostic tool for detection of aggressive breast cancer and for distinguishing such aggressive and invasive cancer from the non-invasive cancer cells attributable to their expressing Id-2 protein.

The aggressive tumor growth can be treated with antisense Id-1 construct and CLDC-based plasmid antisense delivery is a practical way of achieving such delivery.

The Id-2 protein level changes dramatically at different stages of breast development in the opposite direction of the Id-1 protein level. The increase in the level of Id-2 protein is crucial for normal breast development, and breast cancer cells that produce high levels of Id-2 protein do not, or are less likely to, migrate and invade. They will remain localized in the breast, will not metastasize and are therefore easier to treat.

II. Method for Detection, Diagnosis and Prognosis of Breast Cancer

A method for detection of the aggressive and invasive cancer cells or noninvasive cancer cells comprises detection of *Id-1* and/or *Id-2* genes, or their ratio, or Id-1 and/or Id-2 products, or their ratio, as diagnostic markers for detection of metastatic aggressivity of carcinoma. Such detection is useful both for diagnostic and particularly for prognostic purposes in patients.

As earlier noted, Id-1 protein is expressed at elevated

levels in aggressive breast cancer cell lines. These highly aggressive breast cancer cells have lost serum-dependent regulation of the Id-1 gene expression, which results in constitutively high levels of Id-1 protein. Indeed, it appears that the Id-1 protein plays a key role in the malignant progression of a subset of aggressive and invasive human breast cancers.

While Id-1 represents a marker of poor prognosis for invasive and metastatic breast cancer, in contrast Id-2 represents a marker of good prognosis for breast cancer since the breast cancer cells expressing Id-2 will tend to be localized and not metastasized.

A patient found to have breast cancer, but breast cancer in which Id-2 is being expressed, is one for whom the prospect of recovery by simpler and less invasive techniques, such as lumpectomy, is suggested. Such a patient, therefore, likely does not need the more radical treatments, such as mastectomy, radiation or chemotherapy, that would otherwise be recommended for invasive breast cancer when the high expression of Id-1 protein is detected.

III. Methods Suitable For Detection of Id-1/Id-2 Expression Products

In a therapeutic method of this invention described below, the treating physician who has, for example, found tumors/lumps will typically send a breast tissue sample, as a biopsy, to a pathologist for examination and diagnosis.

The examination and classification of the tissue is typically based on a visual inspection of tissue morphology. For example, the pathologist can decide whether the biopsied tissue is an infiltrating or invasive carcinoma or whether it is ductal carcinoma *in situ* (DCIS). Within each of these classifications the pathologist attempts to assign grades of aggressiveness, such as infiltrating Grade 1 carcinoma, which is not overly aggressive, or infiltrating Grade 3 carcinoma

that is very aggressive.

The development of a DCIS into a highly aggressive and metastatic breast tumor involves a series of sequential steps; breast epithelial cells must lose the ability to interact with other cells, acquire the ability to digest the surrounding basement membrane, migrate toward the blood stream, and survive and proliferate in ectopic sites. Invasiveness marks the onset of metastasis, which is a hallmark of often final malignant progression.

For detection of Id-1/Id-2 proteins, the immunohistochemistry analysis using Id-1 antibodies can be used together with Id-2 antibodies, since a determination of both Id-2 and Id-1 expression, or lack of expression for one with respect to the other, will help the treating physician and pathologist determine the type or grade of breast cancer. Thus, determination of Id-1 or Id-2 expression ratio, or the ratio of *Id-1* to *Id-2* gene product such as proteins or mRNA, can be performed by various detection methods known to the art such as immunohistochemistry or *in situ* hybridization.

Where the gene products to be determined are proteins, then the Id-1 and Id-2 proteins can be detected and analyzed, for example, by immunohistochemistry as described in Examples 8 and 10, where anti-serum is directed against the gene product of interest.

Additionally, the presence or absence of a gene product, mRNA, can be detected in accordance with this invention through the use of probes, primers or anti-sense molecules. Such detection utilizes, for example, probes for detecting and/or analyzing Id-1 and Id-2 expression, such as in *in situ* hybridization to detect target mRNA.

Where the Id-1 and Id-2 gene products to be detected are, for example, mRNA, then the detection can be accomplished, for example, with nucleic acid probes. Other means for detecting the presence or absence of the mRNA gene product that are

known and useful can utilize primers and anti-sense molecules.

The DNA of the invention encoding the Id-1 or Id-2 gene or homologues, analogues, or fragments thereof may be used in accordance with the invention to diagnose disease states which are phenotypic of an aberrant Id-1 or Id-2 genotype or of aberrant Id-1 or Id-2 expression.

By way of another example, but not by way of limitation, many tumors may be characterized by a lack of, or excess of, Id-1 or Id-2 activity which may stem from mutations in the Id-1 or Id-2 coding or regulatory sequence.

In both of the examples above, afflicted cells, tissue sections or biopsy specimens may be screened with the *Id-1* or *Id-2* DNA sequences of the invention and isolated *Id-1* or *Id-2* sequenced to determine which mutations in Id-1 or Id-2 are associated with the diseases. The DNAs of the invention may also be used to determine whether an individual carries an aberrant Id-1 or Id-2 gene.

The detection of the aberrant Id-1 or Id-2 DNA is conducted by PCR amplification, from a small tissue sample. Detection of Id-1 or Id-2 product may also be via *in situ* hybridization or immunocytochemistry of pathology or biopsy specimens.

The best mode contemplated for practicing the invention for detection of breast cancer cell aggressivity is to perform assays from biopsied breast tissue for both Id-1 and Id-2 proteins or mRNAs. In practice, one or more of the sections made from an embedded biopsy are tested for Id-1 and for Id-2. The results are then compared for ratios of Id-1 and Id-2, since it appears that Id-1 and Id-2 are inversely correlated. The importance of determining the ratios of Id-1 and Id-2 will be specific for breast tissue and breast cancers, by contrast to other tissues and other cancers, where different ratios may be found.

B. Antibodies

In addition, Id-1 and Id-2 antibodies can be used in a number of other detection methods, since many of the detection methods known in the art that will be useful in detecting Id-1 and Id-2 gene products utilize antibodies.

One aspect of this invention is a method for using Id-1 and Id-2 antibodies where the antibodies will bind to Id-1 and Id-2 proteins, respectively, if present, in a breast, cervical, ovarian, endometrium, squamous cells, prostate or melanoma tissue sample. The presence of bound antibodies can be determined by simple visual examination, or can be detected by other known methods, such as radioactivity or fluorescence.

For the production of antibodies, various host animals may be immunized by injection with the *Id-2* or *Id-1* gene product, or a portion thereof including but not limited to, portions of the *Id-1* or *Id-2* gene product in a recombinant protein. Such host animals may include but are not limited to rabbits, mice, and rats, to name but a few.

Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolccithin, pluronic polyols, polyanions, peptides, oil emulsions, kehole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Id-1 and Id-2 antibodies are commercially available. The commercially available antibodies are typically polyclonal, and bind to both the mouse and human proteins.

Monoclonal antibodies may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described in Nature, 256:495-497 (1975), the human B-cell hybridoma technique, Immunology Today, 4:72 (1983), Proc. Natl. Acad. Sci., 80:2026-2030 (1983) and the EBV-hybridoma technique, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 (1985).

In addition, techniques developed for the production of "chimeric antibodies", Proc. Natl. Acad. Sci., 81:6851-6855 (1984), Nature, 312:604-608 (1984), Nature, 314:452-454 (1985) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described, for example, the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies specific to one of the binding partners.

Antibody fragments which recognize specific epitopes may be generated by know techniques. For example, such fragments include but are not limited to: the $F(ab^1)_2$ fragments which can be produced by pepsin digestion of the antibody molecule and

the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab^1)_2$ fragments. Alternatively, Fab expression libraries may be constructed according to Science, 246:1275-1281 (1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

C. *Id-1* and *Id-2* Genes, Id-1 and Id-2 Protein - Markers for Detection of and Targets for Treatment

Because of their negative correlation and different function in the breast tissue, *Id-1* or *Id-2* genes, Id-1 or Id-1 mRNAs, or Id-1 or Id-2 proteins may each individually be used as a marker for detection and/or prognosis of malignant aggressivity or as a target for gene therapy.

D. Combination of *Id-1* and *Id-2* Genes - Marker and Target

Similarly, a ratio of both genes and/expressed proteins may be advantageously used for diagnosis and/or prognosis of breast cancer cells aggressivity.

E. Prognosis of Breast Cancer

In one aspect of the present invention, a method is provided that is useful in the prognosis of breast cancer.

The method for prognosis comprises detecting expression for an *Id* gene product in breast tissue obtained from a patient, and more preferably by seeking to detect gene products, that is Id-1 and Id-2 proteins or mRNAs. For example, the presence of *Id-2* gene product (protein or mRNA) and the absence of Id-1 gene product, or a relatively larger amount of Id-2 with respect to Id-1, is a prognostic indicator that breast cancer cells in the breast tissue will remain localized.

IV. A Diagnostic Kit for Detection of Breast and Other Types of Cancer Aggressivity

The invention further concerns the detection of Id-1, Id-2 or their ratio with a kit comprising anti Id-1 and/or Id-2 antibodies or *Id-1* and/or *Id-2* probes.

The kit for detection of breast cancer aggressivity is based on a method of using Id-1 and Id-2 antibodies or probes.

The kit typically comprises a detection means for detecting either the Id-1 and/or Id-2 expression product mRNA, or Id-1 and/or Id-2 product. For detection of Id-1 or Id-2 protein, antibodies for Id-1 protein are contacted with breast tissue under conditions allowing the Id-1 antibodies to bind to Id-1 protein, if present. Another sample of the same breast tissue is similarly contacted with antibodies for Id-2 protein under conditions allowing the Id-2 antibodies to bind to Id-2 protein, if present. The presence of bound Id-2 antibodies with the absence of bound Id-1 antibodies is a prognostic indicator that breast cancer cells in the breast tissue are noninvasive and remain localized. The presence of Id-1 antibodies with the absence of Id-2 binding is a prognostic indicator of the presence of aggressive cancer. Quantitating both responses derives a ratio of Id-1/Id-2. The ratio above 1 indicates aggressive cancer. The ratio lower than 1 indicates less aggressive or non-aggressive cancer.

V. Method for Treatment of Breast Cancer

A method for treatment of breast, endometrial, cervical, ovarian, squamous cells or prostate carcinoma or melanoma comprises targeting of *Id-1*, or *Id-2* genes, or a combination thereof, through delivery of antisense transcripts, ribozymes, cationic liposomes, small therapeutically active molecules, drugs, peptides or organic compounds that disrupt *Id-1* interaction with a bHLH transcription factor or enhance *Id-2* gene interaction with a bHLH transcription factor and vice versa, RNA, anti-*Id-1* RNAi causing degradation of homologous *Id-1* mRNAs, Id-2 as a gene or a protein, ITF-2 as a gene or protein, or targeting Id-1 or Id-2 proteins with antibodies or with compounds which either enhance or inhibit their production.

A. Gene Therapy for Treatment

Gene therapy provides a way to manipulate genetic make-up of the cell. There are two general approaches to gene therapy.

The first approach utilizes the introduction into a patient of a vector that inserts into the genetic code a sequence in the case of breast cancer, Id-2 sequence, that replaces the more aggressive Id-1 gene, with the less aggressive Id-2 gene.

The second approach utilizes the genetic code of Id-1 or Id-2 to deliver to the breast cells Id-1 or Id-2 antisense molecules that enter the breast cells and by sequence recognition, selectively inhibit the gene, Id-1 gene in this case, expression.

Both approaches are intended to be within the scope of this invention.

B. Gene Therapy Approaches

A variety of gene therapy approaches may be used in accordance with the invention to modulate expression of the *Id-1* or *Id-2* gene *in vivo*. For example, antisense DNA molecules may be engineered and used to block translation of mRNA *in vivo*.

Alternatively, ribozyme molecules may be designed to cleave and destroy the *Id-1* or *Id-2* mRNAs *in vivo*.

In another alternative, oligonucleotides designed to hybridize to the 5' region of the *Id-1* or *Id-2* gene (including the region upstream of the coding sequence) and form triple helix structures may be used to block or reduce transcription of the *Id-1* or *Id-2* gene.

In yet another alternative, nucleic acid encoding the full length wild-type *Id-1* or *Id-2* message may be introduced *in vivo* into cells which otherwise would be unable to produce the wild-type *Id-1* or *Id-2* gene product in sufficient

quantities or at all.

In a preferred embodiment, the antisense, ribozyme and triple helix nucleotides are designed to inhibit the translation or transcription of *Id-1* with minimal effects on the expression of *Id-2*. In a preferred embodiment, the antisense, ribozyme and triple helix nucleotides are designed to inhibit the translation or transcription of *Id-2* with minimal effects on the expression of *Id-1*. To accomplish this, the oligonucleotides used are designed on the basis of relevant sequences unique to *Id-1* or *Id-2*, i.e., those sequences found in *Id-1* but not in *Id-2* or *Id-2* and not *Id-1*.

For example, and not be way of limitation, the oligonucleotides should not fall within those regions where the nucleotide sequence of both *Id* genes is most homologous.

Moreover, the aggressive propensity of *Id-1* gene in breast cancer cells may be effectively targeted with *Id-1*-antisense construct and the aggressive breast cancer cells may be converted to non-aggressive non-invasive cancer cells.

B. Targeting Delivery Vehicles and Products

The current gene delivery methods can be divided to two classes: viral and non-viral.

a. Viral Vectors

The viral vectors currently used both for target validation and gene therapy are mainly of the following types:

1. Adenoviral vectors, mostly Ad2 and Ad5-based recombinant vectors which may or may not contain targeting elements, either via genetic modification or chemical modification of the viral capsid. It can either be a replication-defective virus or a selectively replicating competent virus.

2. Lentis viral vectors with the same modifications as stated for adenoviral vectors.

3. Adeno-associated viral vectors (AAV).

4. Retroviral vectors.

Among these four, the first two are most commonly used for cancer indications.

b. Non-viral Gene Delivery Vehicles

There are several non-viral based gene delivery systems.

1. One class includes physical devices to facilitate uptake including direct injection of plasmid DNA, gene guns, electroporation, microinjection, electrical pulses, and ultrasound.

2. The other class of non-viral based methods more relevant to systemic delivery are the synthetic gene delivery systems that are defined by their use of:

i) cationic lipids, also called cationic liposomes or lipoplexes; Cationic lipids enter the cell by endocytosis and traverse the cytoplasm through various endocytic compartments. In this process, these complexes are either targeted to lysosomes for degradation, or are released into the cytoplasm. One way to deliver gene to its target is by forming cationic liposome-DNA complex which targets gene expression to vascular endothelial cells, macrophages and tumor cells.

In practice, for example, cationic liposome-*Id-2*-DNA complex is prepared and targeted to carcinoma cells to replace a highly aggressive Id-1 gene with less aggressive Id-2 gene.

ii) polycationic polymers or polyplexes.

3. Another delivery vehicle for targeting of the Id-1 gene is RNA interference (RNAi) process. The RNAi process utilizes a sequence-specific post-transcriptional gene silencing of *Id-1* gene by providing a double-stranded RNA (*Id-1*-dsRNA) that is homologous in sequence to the Id-1 gene. Small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer Id-1-dsRNA are the mediators of sequence-specific Id-1-mRNA degradation.

4. Another type of targeting delivery vehicles are recently newly developed nanotechnologies. There are currently two nanotechnologies developed and available for gene transfers and drug delivery, namely dendritic polymers and micellar nanoparticles. Dendritic polymers, also called dendrimers are polymers suitable and useful for the design and assembly of nanoscale materials. Micellar nanoparticles are unique synthetic lipid vesicles that fuse with cell membrane.

Non-viral based gene delivery systems offer ease of preparation, enhanced DNA packaging capacity and low immunogenecity.

In terms of the type of molecules the gene delivery vehicles can deliver, they include plasmids expressing cDNA of the therapeutic genes (ITF-2 or Id-2, for example in the breast) or the actual therapeutic molecules. Additionally, anti-sense expressing plasmids (Id-1 antisense, for example) or the anti-sense oligonucleotides themselves may be used as a delivery vehicle to target cancer genes. Small molecule inhibitors of Id-1-interacting proteins are also suitable.

The use of antisense DNA and DNA vectors is described, for example, in Clinical Trails of Genetic Therapy with Antisense DNA and DNA Vectors, Ed. Eric Wickstrom, Marcel Decker, Inc. (1998), incorporated by reference.

In conclusion, there are different ways to develop cancer therapeutics using helix-loop-helix proteins as targets. These different ways include, but are not limited to, the ones previously described.

VI. Pharmaceutical Formulations and Compositions

Any of the identified compounds, antisense DNA molecules, antibodies, delivery vehicles, etc., can be administered to a mammal, including a human patient, directly, or in pharmaceutical compositions comprising its admixture with suitable carriers or excipient(s) at doses therapeutically effective to treat or ameliorate a breast, cervical, ovarian,

endometrium, squamous cells and prostate cancer and melanoma.

A therapeutically effective dose refers to that amount of the composition sufficient to result in treatment or amelioration of symptoms associated with aggressive cancer cells. Various techniques for formulation and administration of the compositions of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition.

The products of the invention may be designed or administered for tissue specificity. If the compound comprises a nucleic acid molecule, including those comprising an expression vector, it may be linked to a regulatory sequence which is specific for the target tissue, such as the breast tissue, cervix, ovarian, endometrium, squamous cells, prostate or skin, etc., by methods which are know in the art including those set forth in Ann. Oncol., 5 Suppl 4:59-65 (1994); Gene, 145:305-310 (1994); Surgery, 116:205213 ((1994); Cancer Res., 54:4266-4269; Cancer, 74 (Suppl. 3):1021-1025 (1994); Proc. Nat'l. Acad. Sci. USA, 91:1460-1464; Exp. Hematol., 22:223-230; Prog. Clin. Biol. Res., 388:361-365 (1994). The compounds of the invention may be targeted to specific sites by direct injection to those sites, such as breast, in the case of breast cancer.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to stop aggressive metastatic cancer growth and to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, the

therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 (the dose where 50% of the cells show the desired effects) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population).

The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds which exhibit high therapeutic indices are preferred. The data obtained from the cell culture assays described above and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity.

The dosage may vary within this range depending upon the dosage from employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patent's condition. Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the desired effects.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable

excipients are, in particular, fillers such as sugars, including lactosse, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions and a suitable organic solvent or solvent mixture. Dye stuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticiser, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethlene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered

in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluorethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with the added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions to the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as a sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, liposomes or cationic liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or destran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder

form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulation described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identify of the co-solvent components may be varied.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes, particularly cationic liposomes, and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the

compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

Routes of Administration

Suitable routes of administration may, for example, include oral, rectal, transmucosal, transdermal, or intestinal administration, parenteral delivery, including intramuscular, subcutaneous, intravenous, intraperitoneal or intranasal.

Alternatively, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into an affected area, often in a depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with an antibody specific for affected cells. The liposomes will be targeted to and taken up selectively by the cells.

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention.

EXAMPLE 1

Production of pBabe- Id-1 Retroviral Vector and Virus

This example describes production of pBabe-Id-1 retroviral vector and virus.

The full-length human *Id-1* cDNA was excised from CMV-*Id-1* and cloned into pBabe-puro, a gift from Dr. Hartmut Land, ICRF, London, United Kingdom. Clones in which the *Id-1* cDNA was inserted in the sense orientation (pBabe-*Id-1*) were selected for use.

pBabe-*Id-1* was transfected into the TSA54 packaging cell line (Cell Genesis; Foster City, CA) using calcium phosphate. Twenty-four hours after transfection, culture medium containing infectious virus was harvested twice at 4 hour intervals and was frozen at -80°C. Viral titers were determined by reverse-transcriptase activity. Briefly, thawed aliquots of harvested media were incubated with poly(A) (20 ng/μl), oligo dT (10 ng/μl), and [^{3}H]TTP (0.1 μCi/μl) in reaction buffer (50 mM Tris-HCl, 75 mM Kcl, 0.5 mM EGTA, and 5 mM $MgCl_2$) for 30 minutes at 37°C. The reaction mixture was spotted on Whatman DE81 paper, which was washed with 2X SSC and counted in a scintillation counter. One unit of MMLV reverse transcriptase (Life Technologies, Inc.) was subjected to the same reaction, and the amount of incorporated [^{3}H]TTP was defined as 1 RT unit. The retroviral titer (RT units/ml) was determined by comparing the amount of [^{3}]TTP incorporated by the virus-containing medium with that incorporated by MMLV reverse transcriptase.

EXAMPLE 2

Cell Culture and Retroviral Infection

This example describes cell lines, cell culture conditions and retroviral infection.

Human breast cancer cell lines MCF7, T47D, and MDA-MB-231 were purchased from the American Tissue Culture Collection

(ATCC). Metastatic MDA-MB-435 cells from ATCC were selected for a highly aggressive phenotype by passage in immunodeficient mice. Briefly, cells were injected into nude mice and fast growing tumors were harvested 3-4 weeks later and processed for *in vitro* cultivation. Fibroblasts were eliminated from the culture by differential trypsinization, and the tumor cells were expanded and cryopreserved for future use.

Breast cancer cell lines were grown in DMEM or RPMI 1640 obtained from University of California, San Francisco, containing 10% fetal bovine serum and insulin (5 μg/ml, Sigma). For experiments using serum-free medium, fetal bovine serum was omitted.

Approximately eight RT-units of either pBabe-puro or pBabe-Id-1 retrovirus were mixed with 5 ml of medium containing 4 μg/ml polybrene and were added to T47D cells in 100-mm dishes. Cells expressing the retroviral genes were selected in 0.6 μg/ml puromycin, which killed all of the mock-infected cells within three days, whereas 80 or 30% of the pBabe-puro- or pBabe-Id-1-infected cells, respectively, survived. These puromycin-resistant cells are referred to as T47D-pBO or T47D-Id-1. To establish single-cell clones, the T47D-Id-1 population was plated at 1-2 cells/well in 24-well tissue culture plates. Clones that grew in the wells were expanded.

EXAMPLE 3

RNA Isolation and Northern Analysis

This example describes conditions used for RNA isolation and Northern analysis.

Total cellular RNA was isolated and purified as described in Anal. Biochem., 162:156-159 (1987). Twenty μg were separated by electrophoresis through formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N; Amersham). The membrane was hybridized to a ^{32}P-labeled human *Id-1* cDNA or Id-

2 or β-casein probe according to J. Biol. Chem., 269:2139-2145 (1994) and was washed and exposed to XAR-5 film for autoradiography. The same blot was hybridized to a 28S rRNA probe to control for RNA integrity and quantitation.

EXAMPLE 4

Western Analysis

This example describes conditions used for Western analysis of breast cancer cells.

Cells were lysed in 2X Laemmli buffer and stored at -70°C. Protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, California). Samples (20-30 μg) were separated by SDS-PAGE and were transferred to a Immobilin-P filter (Millipore) by standard methods. The membrane was blocked for 1 hour at room temperature with TBST (20 mM Tris Base, 137 mM NaCl, 3.8 mM, HCl, and 0.1% Tween 20) containing 5% nonfat milk, and incubated with a rabbit polyclonal antibody against human Id-1 or Id-2 (C-20; Santa Cruz Biotechnology) or with a rabbit polyclonal antibody specific for the PR-A and PR-B forms of the Pg receptor (C-20; Santa Cruz Biotechnology) for 1.5 hours. The membrane was washed, incubated with secondary antibody (goat antirabbit IgG-horseradish peroxidase; Santa Cruz Biotechnology), washed again, and developed for enhanced chemiluminescence using the Amersham ECL kit, according to the supplier's instructions.

EXAMPLE 5

Boyden Chamber Invasion Assays

This example illustrates conditions used for Boyden Chamber invasion assays.

Invasion assays were performed in modified Boyden chambers with 8 μm pore filter inserts for 24-well plates (Collaborative Research). Filters were coated with 10-12 μl of ice-cold Matrigel (8 mg/ml protein; Collaborative Research). Cells (80,000 per well) were added to the upper chamber in 200 μl of the appropriate medium containing 0.1%

BSA. Cells were assayed in triplicate or quadruplicate, and the results were averaged. The lower chamber was filled with 300 μl of NIH-3T3 cell-conditioned medium. After a 20 hour incubation, cells were fixed with 2.5% glutaraldehyde in PBS and were stained with 0.5% toluidine blue in 2% Na_2CO_3. Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy.

EXAMPLE 6

[^{3}H]-Thymidine-Labeling

This example describes conditions used for labeling cells with [^{3}H]-thymidine.

Cells cultured on coverslips were given [^{3}H]-thymidine (10 μCi/ml; 60-80 Ci/mmol; Amersham) for the last 16 hours of the experiments, unless otherwise indicated, whereupon they were fixed with methanol/acetone (1:1) and stained with DAPI. [^{3}H]-thymidine-labeling was developed as described previously in Mol. Cell Biol., 18:4577-4588 (1988). The percentage of labeled nuclei was calculated by comparing the number of [^{3}H]-thymidine-labeled nuclei with the number of DAPI-stained nuclei in a given field, using phase contrast and fluorescence microscopy.

EXAMPLE 7

Antisense Oligonucleotide Treatment

This example describes conditions used for antisense oligonucleotide treatment of T47D cells.

Phosphorothiolated oligonucleotides were made by Life Technologies, Inc. The *Id-1* antisense oligonucleotide and nonspecific control oligonucleotide were described in J. Biol. Cheml, 269:2139-2145 (1994). T47D cells were cultured on coverslips in serum-free medium for 2 days. On days 3 and 4, the medium was changed in the morning to serum-free medium containing either E2 (10 nM), or E2 and the oligonucleotides (10 μM). On the evening of day 4, protein was extracted from

one set of dishes, whereas [^{3}H]-thymidine was added to the other set for an additional 16 hours. Cells were fixed on day 5 and assessed for labeled nuclei as described above.

EXAMPLE 8

Immunohistochemistry

This example describes conditions used for immunohistochemical treatment of tumor tissue sections.

Formalin-fixed paraffin-embedded tumor tissue sections obtained from the CPMC patient protein expression in both DCIS and infiltrating Grades 1, 2 and 3 ductal carcinomas.

Slides were de-waxed, re-hydrated, and placed in a container containing 1 liter of 0.01 M citrate buffer (pH 6.0); they were then microwaved at 700 W for 20 minutes, allowed to remain in the hot citrate buffer for 15 minutes, and cooled down in running cold water. The slides were washed in deionized water and incubated in 10% nonfat dry milk for 30 minutes at room temperature, washed in TBS, and incubated with 1 μg/ml of anti Id-1 antibody overnight at 4°C. Control slides were incubated with rabbit immunoglobulins. The slides were washed in TBS and incubated with biotinylated swine antirabbit $F(ab')_2$ fragments (I:400) for 30 minutes. After washing in TBS, endogenous peroxidase was visualized by incubating in 0.5 mg/ml diaminobenzidine-4-HCl and 0.03% hydrogen peroxide in TBS for three minutes. The slides were washed in TBS and water before mounting.

EXAMPLE 9

Manipulation of Id-2 Expression in Breast Cells

This example describes methods used for manipulation of Id-2 expression in breast cells.

Id-2 cDNA was digested with XbaI and HindIII to isolate a 1.2 kb fragment. The viral LXSN vector that was used for the mouse Id-2 cDNA has already been digested with EcoRI, blunted with T4 DNA polymerase and dephosphorylated with CIAP. The Id-2 fragment was similarly blunted with T4 DNA

polymerase, was inserted inside the dephosphorylated vector, and the ligation product transformed into Top-10 cells. To identify the clones with sense or anti-sense orientation, digestion of the recovered plasmids was performed with either NcoI or BstEII enzymes, and the size of the expected fragments determined on ethidium bromide agarose gels. The viral vectors was then packaged in TSA-54 cells (Cell Genesis; Foster City, CA). Mammary epithelial cells were infected with control, Id-2 sense or Id-2 antisense vectors and selected with neomycin. One to two weeks after infection, resistant colonies were pooled and expanded.

EXAMPLE 10

Id-2 Protein Expression in Tumor Biopsies

This example describes studies performed to demonstrate Id-protein expression in tumor biopsies.

Breast samples have been obtained from patients undergoing tumorectomies. In order to maintain the integrity of the tissue, paraffin embedded sections were used instead of frozen sections. Tissues were fixed overnight at 4°C in PBS, pH 7.2, containing 4% paraformaldehyde, dehydrated by graded alcohol and finally embedded in paraffin.

Id-2 expression is studied in a representative number of *in situ* and invasive breast tumors. As for Id-1, a sample size of 30 ductal carcinomas in situ as well as 30 invasive Grade I and 30 invasive Grade 3 tumor tissues are used.

A specific rabbit anti-Id-2 antibody obtained from Santa Cruz Biotechnology (C-20) is used for immunohistochemistry experiments. Slides are dewaxed, rehydrated and placed in a container containing citrate buffer (pH 6.0), microwaved, allowed to remain in the hot citrate buffer for 15 min, and cooled down in running cold water. The slides are washed in deionized water and incubated in 10% non fat dry milk, washed in TBS and incubated with 1 µg/ml of anti Id-2 antibody overnight at 4°C. Control slides are incubated with rabbit

immunoglobulin, washed in TBS and incubated with biotinylated swine anti-rabbit F(ab)'2 (1:400). The slides are then washed in TBS and incubated with 1:500 streptavidin-horse radish peroxidase. Peroxidase is visualized by incubating in 0.5 mg/ml diaminobenzidine-4HCl and 0.03% hydrogen peroxide.

It is Claimed:

1. A diagnostic and prognostic method useful for detection of aggressive, metastatic and invasive cells in breast, cervical, ovarian, endometrium and squamous, prostate and melanoma cells cancer tissue, comprising steps:

a) detecting expression for an *Id-1* or *Id-2* gene product in breast tissue obtained from a patient;

b) evaluating results obtained in step (a) wherein *Id-1* gene product expression is a prognostic indicator that cancer cells in the cancer tissue are aggressive and metastatic and *Id-2* gene expression product is a prognostic indicator that cancer cells in the cancer tissue are non-invasive.

2. The method of claim 1 wherein the expression of *Id-1* gene product to the expression of *Id-2* product is defined as a ratio of Id-1 product to Id-2 product, wherein a high ratio indicates aggressive and metastatic cancer and a low ratio indicates non-invasive localized cancer.

3. The method of claim 2 wherein the expression of *Id-1* or *Id-2* gene is detected as mRNA or Id-1 or Id-2 protein.

4. The method of claim 3 wherein the mRNA is detected using oligonucleotide probes, primers or antisense sequences.

5. The method of claim 4 wherein the mRNA is detected with a Northern analysis.

6. The method of claim 3 wherein the Id-1 or Id-2 protein is detected with Id-1 or Id-2 antibodies, immunohistochemically or radiographically.

7. The method of claim 3 wherein the proteins are detected with a Western analysis.

8. The method of claim 3 wherein the Id-1 or Id-2 mRNA, Id-1 or Id-2 protein is detected as a marker for breast cancer prognosis.

9. The method of claim 1 further detecting aggressive metastatic and invasive prostate cancer and melanoma.

10. The method of claim 9 wherein the prostate cancer aggressiveness and invasiveness is detected as high Id-2 expression product and low Id-1 expression product in aggressive prostate cancer and melanoma.

11. A method for using Id-1 or Id-2 antibodies for detection and prognosis of breast cancer, comprising steps:

a) providing antibodies specific for Id-1 protein and antibodies specific for Id-2 protein;

b) contacting the Id-1 antibodies with a first sample of breast tissue and contacting the Id-2 antibodies with a second sample of breast tissue under conditions allowing the antibodies to bind to protein, if present, and

c) comparing the amount of bound antibody in each of the first and second samples.

12. The method as in claim 11 wherein the presence of bound antibodies is determined by visual examination.

13. A kit useful for the diagnosis or prognosis of breast cancer, comprising:

a) a first quantity of antibodies specific for Id-1 protein;

b) a second quantity of antibodies specific for Id-2 protein; and,

c) a means for performing an assay wherein the first quantity of Id-1 antibodies is contacted with Id-1 protein in a breast tissue, and the second quantity of Id-2 antibodies is adapted with Id-2 protein in a breast tissue, and wherein, if present, the Id-1 antibodies form an Id-1 protein/antibody complex and the Id-2 antibodies form an Id-2 protein/antibody complex and wherein the Id-1 protein/antibody complex and the Id-2 protein/antibody complex quantities are in a determinable ratio effective for predicting either invasiveness or non-invasiveness of the breast cancer.

14. A method for treatment and amelioration of a breast, cervical, ovarian, endometrial, squamous cells, prostate cancer and melanoma in a patient comprising a step of:

a) targeting Id-1 or Id-2 gene expression with a delivery vehicle comprising a product which affects positively or negatively Id-1 or Id-2 expression.

15. The method of claim 14 wherein said cancer is breast, cervical, ovarian, endometrium or squamous cells cancer.

16. The method of claim 15 wherein the product is an antisense transcript, ribozyme, a molecule that disrupts Id-1 interaction with a transcription factor or enhances Id-2 interaction with a transcription factor, RNAi, ITF-2 gene or protein.

17. The method of claim 16 wherein the molecule that disrupts Id-1 or enhances Id-2 interaction is a peptide, pharmaceutical agent or an organic compound.

18. The method of claim 17 wherein the delivery vehicle is an adenoviral, adeno-associated viral, lentis viral or retroviral vector, a cationic liposome, polycationic polymer or polyplex, a pharmaceutically acceptable composition, or a device which facilitates a delivery of such delivery vehicle.

19. The method of claim 14 wherein said cancer is prostate cancer and melanoma.

20. The method of claim 19 wherein the product is an antisense transcript, ribozyme, a molecule that enhances Id-1 interaction with a transcription factor or disrupts Id-2 interaction with a transcription factor, RNAi.

21. The method of claim 20 wherein the molecule that enhances Id-1 or disrupts Id-2 interaction is a peptide, pharmaceutical agent or an organic compound.

22. The method of claim 21 wherein the delivery vehicle is adenoviral, adeno-associated viral, lentisviral or retroviral, vector, cationic liposome, polycationic polymer or polyplex, pharmaceutically acceptable composition, or a device which facilitates a delivery of such delivery vehicle.

23. The method of claim 22 wherein said cancer is prostate cancer or melanoma.

FIG. 1

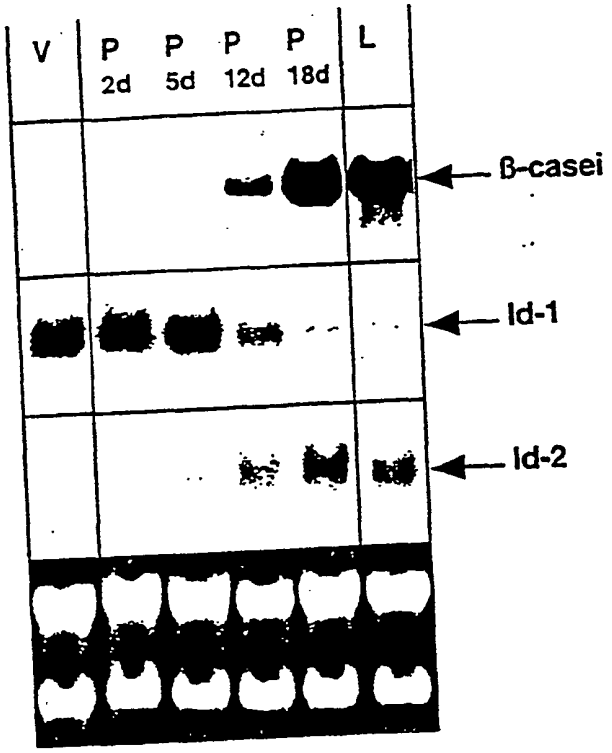

V
P 2d
P 5d
P 12d
P 18d
L
ß-casei
Id-1
Id-2

FIG. 2

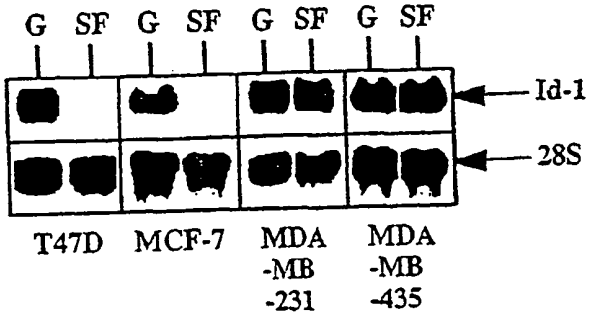

G SF G SF G SF G SF
Id-1
28S
T47D
MCF-7
MDA -MB -231
MDA -MB -435

FIG. 3

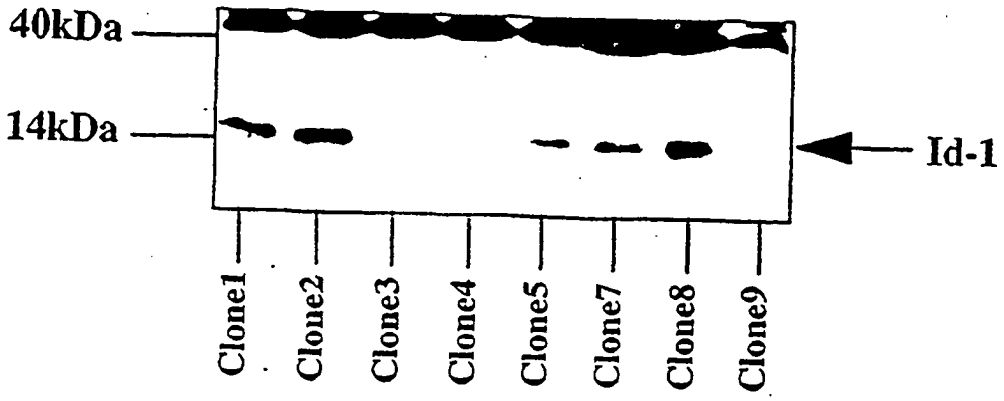


FIG. 4

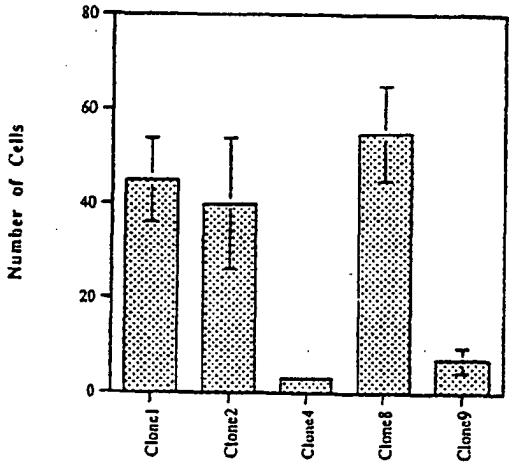


FIG. 5

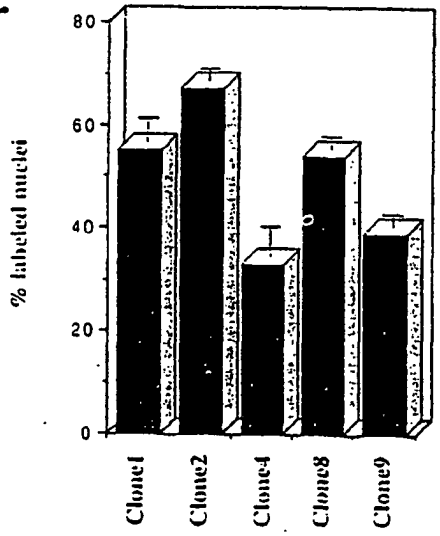


FIG.6 A

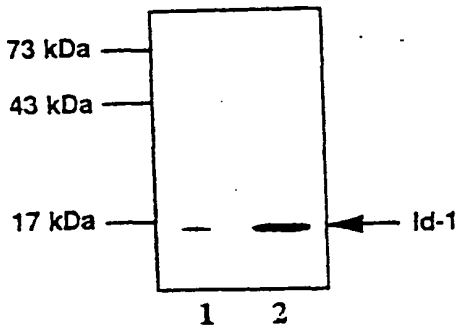
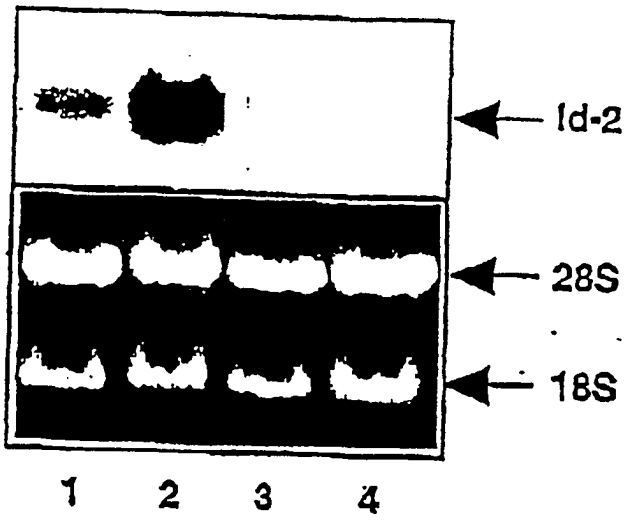


FIG.6 B

FIG. 7



lanes Cancer cell lines

1: T47D

2: MCF7

3: MDA-MB-231

4: MDA-MB-436

FIG. 8

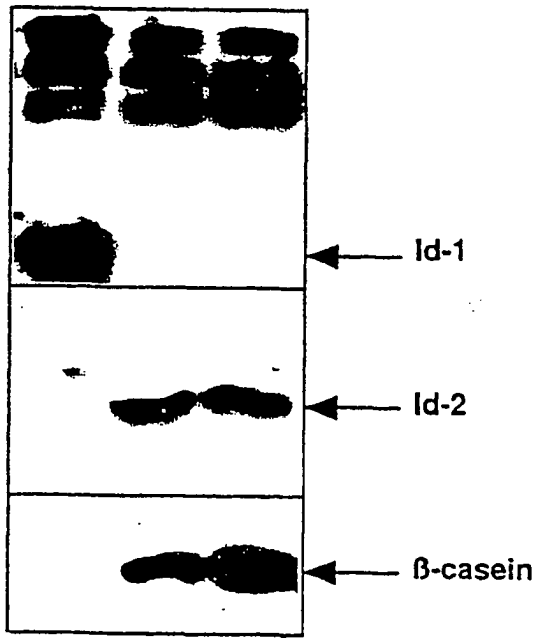


FIG. 9

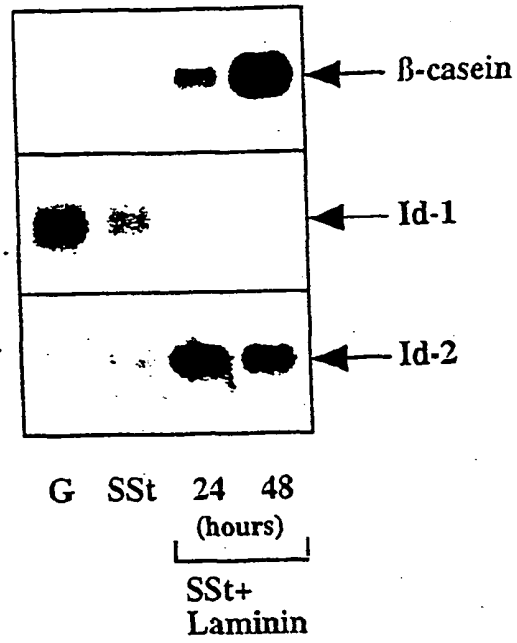


FIG. 10A

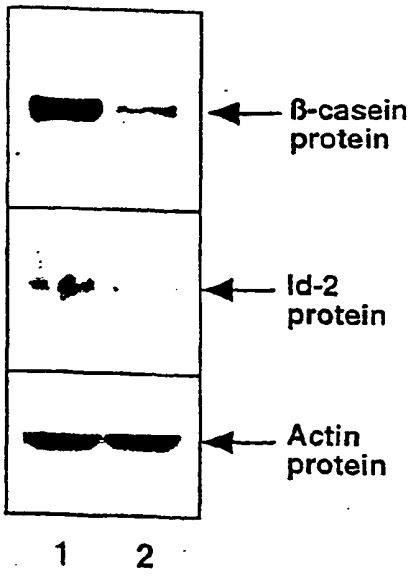


FIG 10B

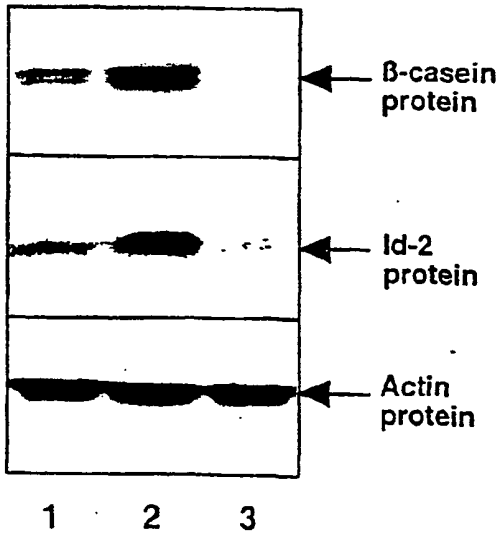


FIG. 11

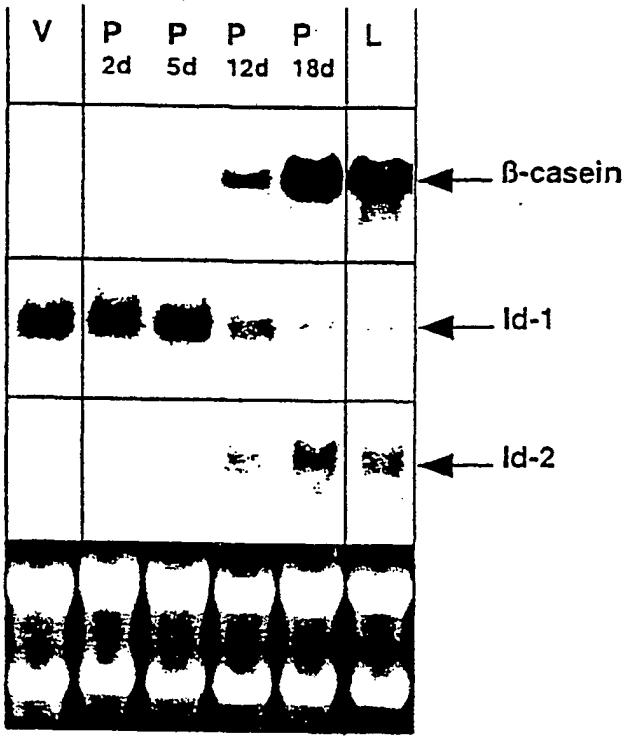


FIG. 12A

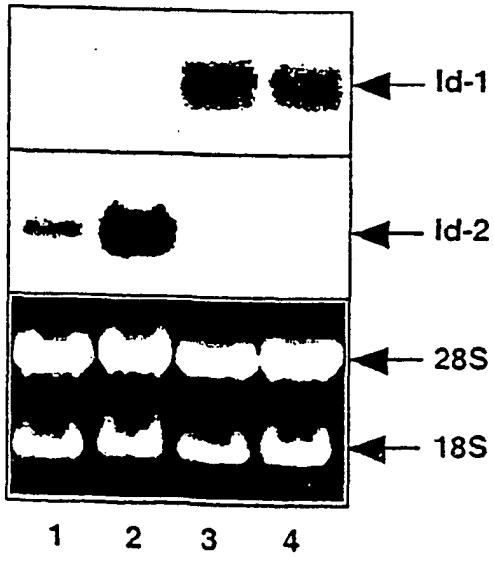


FIG. 12B

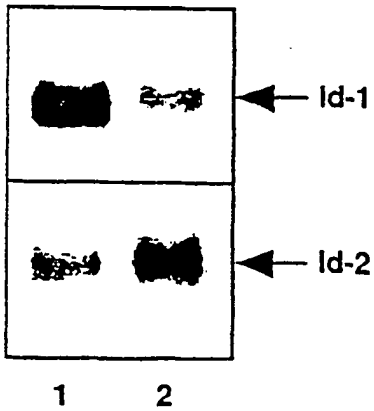
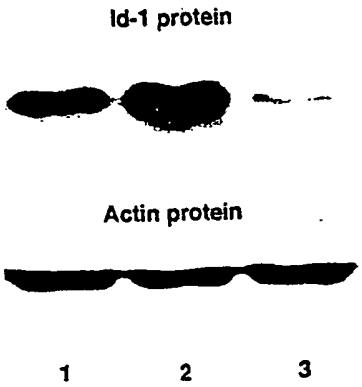


FIG. 13



1: MDA-MB436 control
2: MDA-MB436 Id1 sense
3: MDA-MB436 Id1 AS

FIG. 14

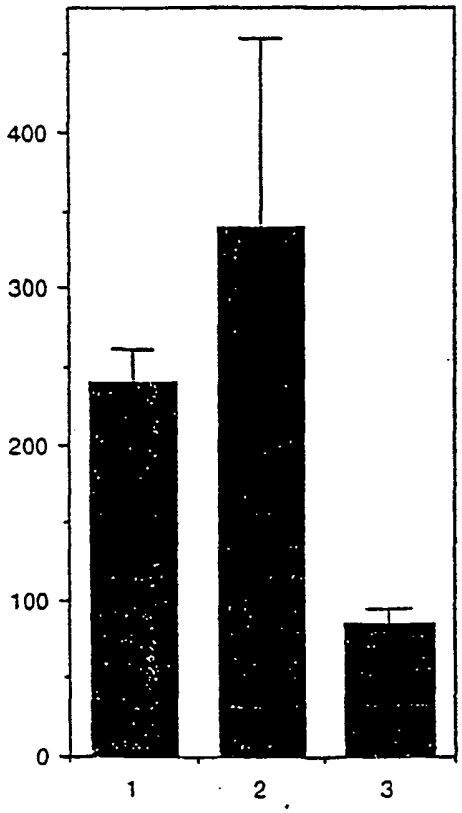
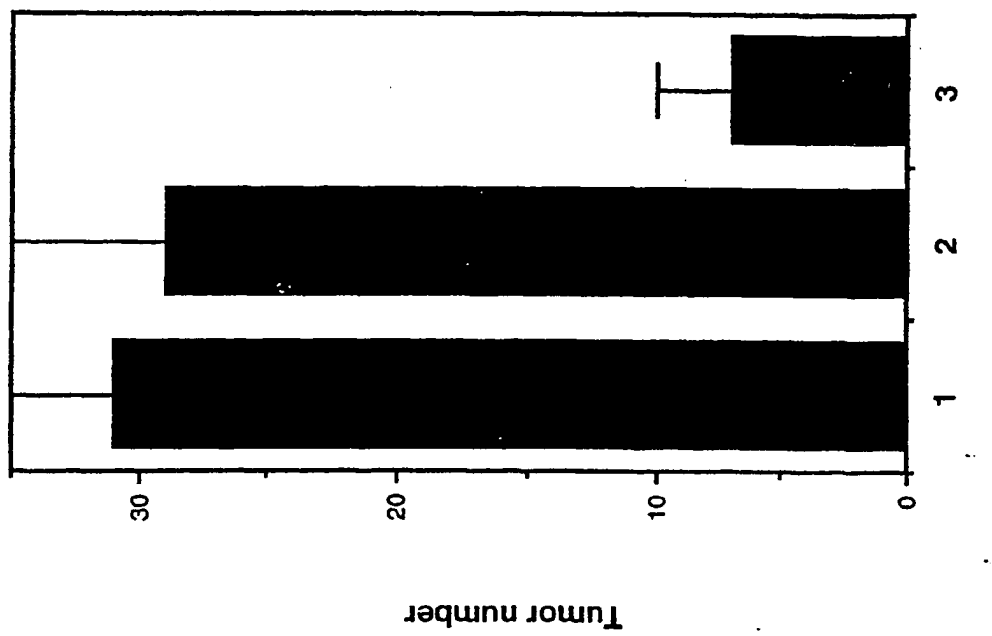


FIG. 15

Tumor number in 4T1/BalbC mice treated with various constructs



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/28811

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/00, 1/68; G01N 33/53; A01N 37/18, 43/04, 61/00; C07H 21/04

US CL :435/4, 6, 7.1; 514/1, 2, 44; 536/24.3, 24.31, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6, 7.1; 514/1, 2, 44; 536/24.3, 24.31, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, BIOSIS, CAPLUS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	LIN et al. A Role for Id-1 in the Aggressive Phenotype and Steroid Hormone Response of Human Breast Cancer Cells. Cancer Research. 01 March 2000, Vol. 60, pages 1332-1340, especially page 1332.	1, 9 14-23
Y	DESPREZ et al. A Novel Pathway for Mammary Epithelial Cell Invasion Induced by the Helix-Loop-Helix Protein Id-1. Molecular and Cellular Biology. August 1998, Vol. 18, No. 8, pages 4577-4588, especially page 4577.	1, 9, 14-23
X, P	TAKAI et al. Id1 Expression is Associated with Histological Grade and Invasive Behavior in Endometrial Carcinoma. Cancer Letters. April 2001, vol. 165, pages 185-193, especially page 185.	1, 9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 NOVEMBER 2001

Date of mailing of the international search report

13 FEB 2002

Name and mailing address of the ISA/US
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SHIN-LIN CHEN

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/28811

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/28811

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-5 and 8-10, in part, drawn to a diagnostic and prognostic method for detection of aggressive, metastatic and invasive cells in cancer tissues, such as breast, endometrium etc. by detecting the expression of an Id-1 or Id-2 mRNA, or by the ratio of Id-1 expression to Id-2 expression via using oligonucleotide probes, primers, or antisense sequences.
Group II, claim(s) 1-3 and 6-13, in part, drawn to a diagnostic and prognostic method for detection of aggressive, metastatic and invasive cells in cancer tissues, such as breast, endometrium etc. by detecting the expression of an Id-1 or Id-2 protein, or by the ratio of Id-1 expression to Id-2 expression via using antibodies against either Id-1 or Id-2.
Group III, claim(s) 14-23, in part, drawn to a method for treatment and amelioration of cancers, such as breast cancers, endometrial, prostate cancers etc., by targeting Id-1 or Id-2 gene expression with antisense transcript.
Group IV, claim(s) 14-23, in part, drawn to a method for treatment and amelioration of cancers, such as breast cancers, endometrial, prostate cancers etc., by targeting Id-1 or Id-2 gene expression with ribozyme.
Group V, claim(s) 14-23, in part, drawn to a method for treatment and amelioration of cancers, such as breast cancers, endometrial, prostate cancers etc., by targeting Id-1 or Id-2 gene expression with peptide or pharmaceutical agent containing said peptide.
Group VI, claim(s) 14-23, in part, drawn to a method for treatment and amelioration of cancers, such as breast cancers, endometrial, prostate cancers etc., by targeting Id-1 or Id-2 gene expression with organic compound or pharmaceutical agent containing said organic compound.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The feature that is common to groups I-VI is Id-1 or Id-2. However, Desprez et al. points out that Id genes encode a small family of proteins that are inhibitors of the basic helix-loop-helix (bHLH) transcription factors. Four Id genes, Id-1 to Id-4, are known where Id-1 and Id-3 are nearly ubiquitously expressed, whereas Id-2 and Id-4 have a more restricted expression pattern, and Id-2 can be physically associated with retinoblastoma tumor suppressor protein pRb (e.g. page 4578, left column). Thus, no common feature is contributed over the prior art. Further, ribozyme is the RNA enzyme that has tRNA or rRNA as substrate. Antisense transcript, ribozyme, peptide, and organic compound of groups III-VI, and mRNA and protein of groups I and II are different products having different chemical structures and biological functions. Groups I-VI are drawn to materially different methods having different process steps, reagents and doses used, and responsive variables. Therefore, groups I-VI do not relate to a single inventive concept under PCT Rule 13.1.